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Boosting genome editing in plants with single transcript unit surrogate reporter systems

Xu Tang[1,2,3,4,8], Qiurong Ren[3,5,8], Xiaodan Yan[2,3], Rui Zhang[4], Li Liu[4], Qinqin Han[4], Xuelian Zheng[1,4], Yiping Qi[6,7,*], Hongyuan Song[2,3,*] and Yong Zhang[1,2,3,4,*]

[1]Chongqing Key Laboratory of Plant Resource Conservation and Germplasm Innovation, Integrative Science Center of Germplasm Creation in Western China (Chongqing) Science City, School of Life Sciences, Southwest University, Chongqing 400715, China

[2]Key Laboratory of Agricultural Biosafety and Green Production of Upper Yangtze River, Ministry of Education, Chongqing 400715, China

[3]College of Horticulture and Landscape Architecture, Southwest University, Chongqing 400715, China

[4]Department of Biotechnology, School of Life Sciences and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu 610054, China

[5]School of Synbiology, School of Life Science, Shanxi University, Taiyuan 030006, China

[6]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD 20742, USA

[7]Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD 20850, USA

[8]These authors contributed equally to this article.

***Correspondence: Yong Zhang (zhangyong916@swu.edu.cn), Hongyuan Song (yuahs@swu.edu.cn), Yiping Qi (yiping@umd.edu)**

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ABSTRACT

CRISPR–Cas-based genome editing holds immense promise for advancing plant genomics and crop enhancement. However, the challenge of low editing activity complicates the identification of editing events. In this study, we introduce multiple single transcript unit surrogate reporter (STU-SR) systems to enhance the selection of genome-edited plants. These systems use the same single guide RNAs designed for endogenous genes to edit reporter genes, establishing a direct link between reporter gene editing activity and that of endogenous genes. Various strategies are used to restore functional reporter genes after genome editing, including efficient single-strand annealing (SSA) for homologous recombination in STU-SR–SSA systems. STU-SR–base editor systems leverage base editing to reinstate the start codon, enriching C-to-T and A-to-G base editing events. Our results showcase the effectiveness of these STU-SR systems in enhancing genome editing events in the monocot rice, encompassing Cas9 nuclease-based targeted mutagenesis, cytosine base editing, and adenine base editing. The systems exhibit compatibility with Cas9 variants, such as the PAM-less SpRY, and are shown to boost genome editing in *Brassica oleracea*, a dicot vegetable crop. In summary, we have developed highly efficient and versatile STU-SR systems for enrichment of genome-edited plants.

Key words: CRISPR–Cas9, single transcript unit, STU, surrogate reporter, SR, single strand annealing, SSA, cytosine base editing, adenine base editing

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INTRODUCTION

Genome editing has become a powerful tool for modifying plant genomes, offering significant potential for plant genomics research and crop genetic improvement (Zhang et al., 2019). Among these technologies, the CRISPR–Cas genome editing system has gained considerable attention due to its efficiency, precision, and easy to use. CRISPR–Cas systems such as Cas9 or Cas12a (Tang and Zhang, 2023), guided by guide RNAs (gRNAs), can target specific positions in the genome, enabling precise editing of targeted genes. Over the years, genome editing techniques have continued to evolve and expand. Two revolutionary editing technologies have emerged in the field of genome editing: base editing and prime editing (Komor et al., 2016; Gaudelli et al., 2017; Anzalone et al., 2019). Base editing enables direct conversion of one base to another, whereas prime editing enables base transition, transversion, and precise

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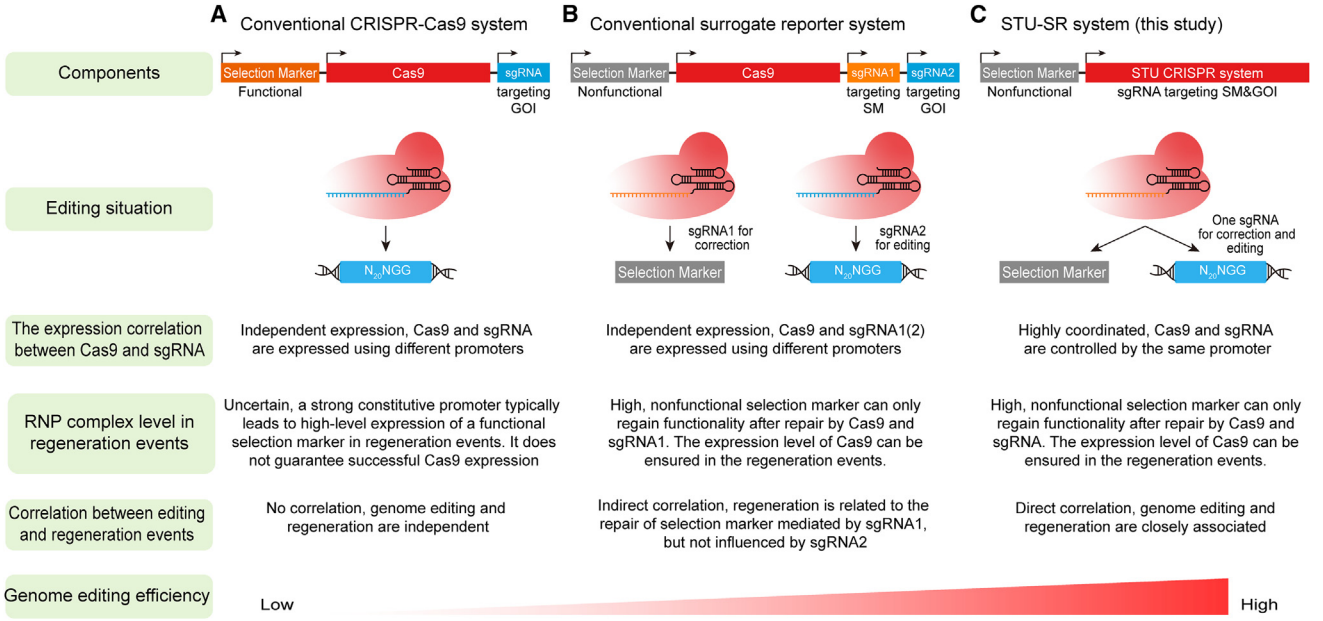


Figure 1. Comparison of three CRISPR–Cas9 editing systems.

(A) In a conventional CRISPR–Cas9 expression system, the plant selection marker, Cas9, and sgRNAs are driven by separate promoters. There is little correlation between editing and regeneration events.

(B) In a conventional surrogate reporter system, CRISPR–Cas9-mediated genome editing events can be enriched because Cas9 expression and activity are selected during plant regeneration. However, there is only an indirect correlation between editing and regeneration events.

(C) In the single transcript unit surrogate reporter (STU-SR) system, both Cas9 and sgRNA are expressed under a single RNA polymerase II promoter. Editing at the reporter gene and at the endogenous target gene is performed by the same sgRNA. There is a direct correlation between editing and regeneration events. Hence, STU-SR systems are stringent and powerful in enriching genome editing events.

short indels without double-strand breaks (DSBs) or a donor repair template (Anzalone et al., 2019). The introduction of these technologies further broadens the scope of genome editing in plants, such as fine-tuning of plant gene expression (Tang and Zhang, 2023; Zhou et al., 2023).

Despite the significant breakthroughs achieved with CRISPR–Cas technologies, there are still challenges that limit their widespread application in plants. One such challenge is that editing efficiency may vary significantly with different gRNAs under different conditions. This instability of editing efficiency complicates the screening of editing events and increases the risk of experimental failure. High genome editing efficiency based on CRISPR–Cas requires optimal delivery and expression of editing reagents, typically via methods such as *Agrobacterium*-mediated transformation. These experiments often involve the use of selectable marker genes (typically conferring resistance to antibiotics or herbicides such as kanamycin, hygromycin, Basta, etc.) to select for transgenic events. These transgenic marker genes can help researchers identify which cells or tissues have successfully received the constructs, but they do not directly reflect the editing status of the target gene. Therefore, researchers often need to further screen the transformation events to determine which events have undergone the desired gene edits. As a result, if the CRISPR–Cas expression levels are low or the editing efficiency at the target site is low in the transgenic events, then it becomes challenging to obtain editing events within the transgenic population. Unfortunately, this happens to be the case for the conventional CRISPR–Cas9 system, in which the selection marker, Cas9, and single guide RNA (sgRNA) cassettes are driven by separate promoters (Figure 1A) (Hassan et al., 2021).

Previously, surrogate reporters were demonstrated to enrich cells with targeted mutations by nucleases such as zinc-finger nucleases and TAL-effector nucleases (Kim et al., 2011). Later, this strategy was applied to enrich CRISPR–Cas9-based genome editing events in mammalian cells (Ramakrishna et al., 2014; Liao et al., 2015). More recently, similar surrogate reporter systems were established in plants to enrich genome editing events by base editing (Xu et al., 2020b), prime editing (Xu et al., 2020a; Li et al., 2020), or Cas9-mediated mutagenesis via a viral vector system (Tian et al., 2022). In these surrogate reporter approaches, dedicated sgRNAs were used to target the broken surrogate reporter genes, while different sgRNAs were used to edit the endogenous genes of interest. Compared with the conventional CRISPR–Cas9 expression system (Figure 1A), these surrogate reporter systems allow for the selection of Cas9 expression because only successful editing of the broken marker genes will result in regenerated plants (Figure 1B). However, because different sgRNAs are used to edit surrogate reporter genes and endogenous genes, these reporter systems only select for events with high CRISPR–Cas expression levels but do not necessarily detect high genome editing activity at the endogenous target sites (Figure 1B). Hence, such surrogate reporter systems fall short in directly reporting genome editing activity for the genes of interest, highlighting a need for more integrated and efficient systems.

Addressing this gap, we introduce single transcript unit surrogate reporter (STU-SR) systems, a leap forward in CRISPR–Cas9 technology (Tang et al., 2016; Tang et al., 2019). Unlike preceding methods, STU-SR systems use identical sgRNAs for both the reporter and target genes, directly linking reporter gene editing with the modification of endogenous genes. This design not only simplifies the selection process by ensuring that successful plant regeneration is intrinsically tied to effective genome editing but also enhances overall efficiency by coordinated expression of Cas9 and sgRNAs under a single RNA polymerase II promoter (Figure 1C). Tailored for versatility across various plant species (Figure 1C), the streamlined vector construction of the STU-SR system markedly improves user accessibility for diverse applications in plant biotechnology. Demonstrated to significantly boost genome editing outcomes in both monocot (rice) and dicot (*Brassica oleracea*) plants, these systems represent a significant advance in our ability to precisely edit plant genomes. These innovative STU-SR systems thus improve editing efficiency and reduce the effort required to screen for genome-edited plants. This approach opens new possibilities for improving precise genome editing that can go beyond plants.

RESULTS

Establishment of an STU-SR–SSA system for enriching CRISPR–Cas9 editing events

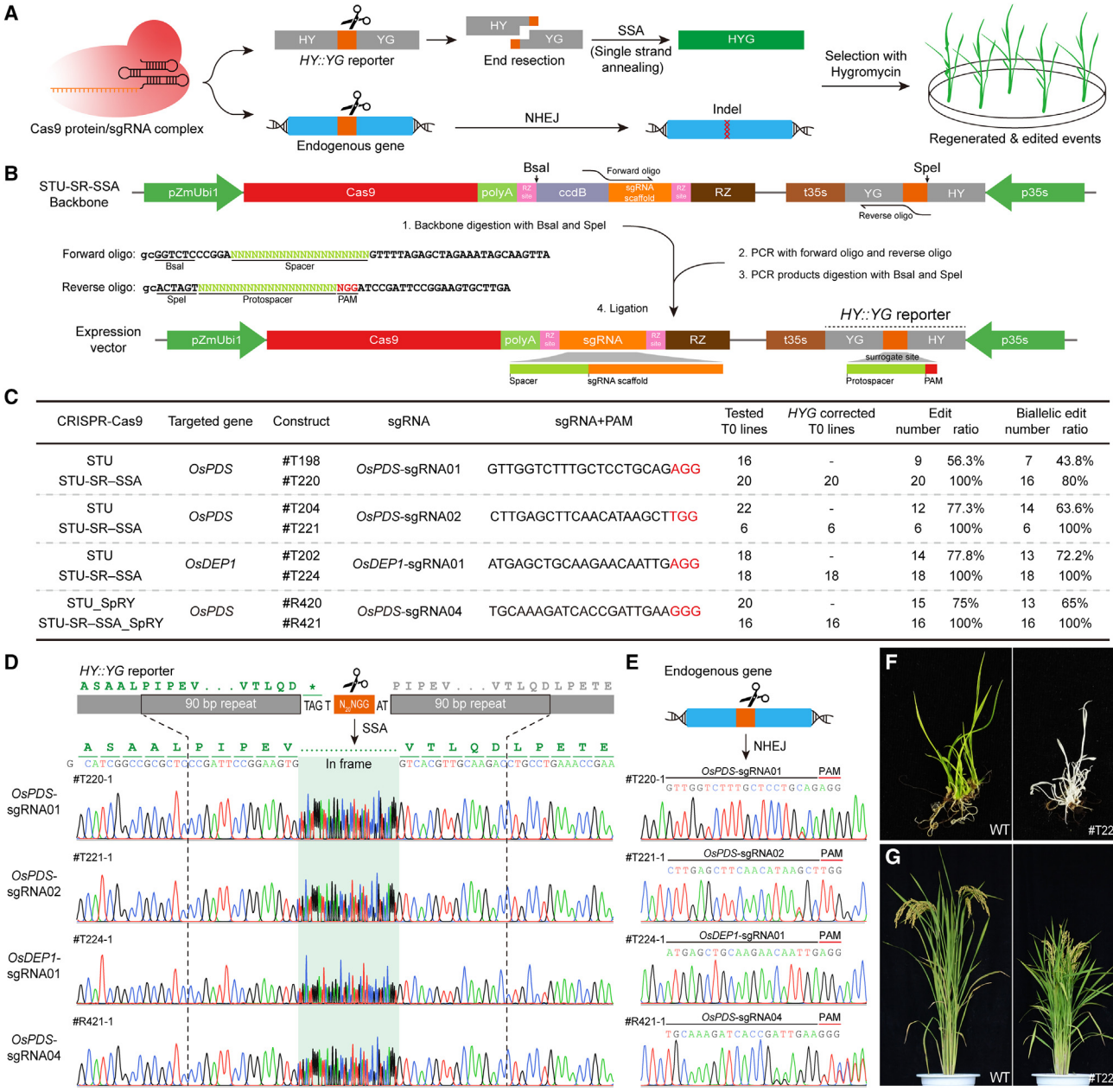
To effectively enrich CRISPR–Cas9 editing events, we tested an STU-SR strategy using sgRNAs designed to target endogenous genes of interest to edit the surrogate reporter genes. Specifically, we used a broken reporter configuration that requires single-strand annealing (SSA)-based homologous recombination to reconstitute a functional reporter, such as a hygromycin phosphotransferase reporter gene (*HYG)* (Figure 2A). SSA was chosen because it is a very efficient homologous recombination repair mechanism in both dividing and non-dividing cells, likely owing to its independence of an exogenous repair template (Puchta, 2005; Roth et al., 2012; Zhang et al., 2013). Initially, we tested this concept in rice by inserting the endogenous target site with the protospacer adjacent motif (PAM), called the surrogate site, within the *HYG* gene, flanked by 90-bp homologous sequences, to construct the *HY::YG* surrogate reporter gene. When the *HY::YG* surrogate reporter gene is cleaved by sgRNAs, it can be restored to a complete *HYG* coding sequence via SSA, leading to the expression of functional *HYG*. Simultaneously, the sgRNA targeting the endogenous gene may induce mutations at the target gene of interest. This enables the enrichment of editing events, achieved by selecting hygromycin-resistant plants (Figure 2A). To construct the STU-SR–SSA vector in rice, we used a tail-to-tail design of the STU Cas9 expression unit and the *HY::YG* reporter expression unit (Figure 2B). This design enables simultaneous cloning of sgRNAs and their target sites into the STU Cas9 expression unit and the *HY::YG* reporter expression unit, respectively. These corresponding DNA fragments can be amplified and ligated into the STU-SR backbone vector after enzymatic digestion with BsaI and SpeI, resulting in the STU-SR expression vectors (Figure 2B).

To test the effectiveness of the STU-SR–SSA system, we compared the editing efficiency of the STU-SR–SSA system with that of the STU CRISPR–Cas9 control system at three endogenous gene target sites in stable transgenic rice plants (Figure 2C). The editing efficiencies for the STU system at the *OsPDS*-sgRNA01, *OsPDS*-sgRNA02, and *OsDEP1*-sgRNA01 loci were 56.3%, 77.3%, and 77.8%, respectively. By contrast, the STU-SR–SSA system achieved 100% editing efficiency at all three sites, representing a 28.5%–77.6% enhancement of editing efficiency compared with the STU system. The biallelic editing efficiencies at these three loci increased from 43.8%–72.2% to 80%–100%, with *OsPDS*-sgRNA02 and *OsDEP1*-sgRNA01 both reaching 100% biallelic editing efficiency (Figure 2C; Supplemental Figure 1). Sanger sequencing confirmed successful editing at both the *HY::YG* reporter gene and the endogenous gene loci (Figure 2D and 2E). *OsPDS* and *OsDEP1* biallelic mutant plants exhibited photobleaching and dwarf phenotypes, respectively (Figure 2F and 2G), consistent with our previous report on the knockout phenotypes of both genes (Lowder et al., 2015).

Previously, we demonstrated that SpRY could achieve PAM-less genome editing in plants (Ren et al., 2021b). However, the overall editing efficiency of SpRY is lower than that of the wild-type SpCas9, likely owing to its PAM-less nature and self-editing when delivered in DNA constructs (Walton et al., 2020; Ren et al., 2021b). It is very appealing to further improve the SpRY expression system for more robust editing outcomes in plants. We investigated whether the STU-SR–SSA system could enhance the editing efficiency of SpRY. The *OsPDS*-sgRNA04 locus was used for testing with stable rice transformation, and the STU-SR–SSA system increased editing efficiency by 33.3% compared with that of the STU system (100% vs. 75%) and enhanced biallelic editing efficiency by 53.8% (100% vs. 65%) (Figure 2C; Supplemental Figure 2). Similarly, Sanger sequencing confirmed successful editing at both the *HY::YG* reporter gene and the endogenous target gene, *OsPDS* (Figures 2D and 2E). These results demonstrate that the STU-SR–SSA system works effectively with Cas9 and its variants for improved genome editing in rice.

Enrichment of genome editing events independent of T-DNA copy number

During *Agrobacterium*-mediated stable transformation, integration of one or more T-DNA copies can occur (Lee and Gelvin, 2008; De Buck et al., 2009; Jupe et al., 2019). When a single T-DNA copy is inserted, the *HY::YG* reporter gene should be corrected, resulting in a single band in the PCR detection (Figure 3A). However, when two or more T-DNA copies are integrated, the *HY::YG* reporter genes may experience the correction of one or multiple copies or even all copies, corresponding to one or two bands in the PCR detection (Figure 3A). We performed PCR detection on eight randomly selected T0 plants per STU-SR–SSA construct. At the *OsPDS*-sgRNA04 site edited by SpRY, two plants (lines 1 and 5) showed a single *HYG* band, exhibiting complete correction of the *HY::YG* reporter gene(s) (Figure 3B). Four plants (lines 2, 3, 6, and 7) showed both *HYG* and *HY::YG* bands of nearly equivalent intensity, suggesting that these lines carried two copies of the



CRISPR-Cas9	Targeted gene	Construct	sgRNA	sgRNA+PAM	Tested T0 lines	*HYG* corrected T0 lines	Edit number	Edit ratio	Biallelic edit number	Biallelic edit ratio
STU	*OsPDS*	#T198	*OsPDS*-sgRNA01	GTTGGTCTTTGCTCCTGCAGAGG	16	-	9	56.3%	7	43.8%
STU-SR–SSA		#T220			20	20	20	100%	16	80%
STU	*OsPDS*	#T204	*OsPDS*-sgRNA02	CTTGAGCTTCAACATAAGCTTGG	22	-	12	77.3%	14	63.6%
STU-SR–SSA		#T221			6	6	6	100%	6	100%
STU	*OsDEP1*	#T202	*OsDEP1*-sgRNA01	ATGAGCTGCAAGAACAATTGAGG	18	-	14	77.8%	13	72.2%
STU-SR–SSA		#T224			18	18	18	100%	18	100%
STU_SpRY	*OsPDS*	#R420	*OsPDS*-sgRNA04	TGCAAAGATCACCGATTGAAGGG	20	-	15	75%	13	65%
STU-SR–SSA_SpRY		#R421			16	16	16	100%	16	100%

Figure 2. Efficient gene editing in rice by the STU-SR–SSA system.

(A) Enrichment diagram depicting the implementation of the STU-SR–SSA system for targeted gene editing in rice.

(B) Schematic illustration of the stepwise cloning strategy used to construct the rice STU-SR–SSA vector for targeted mutagenesis.

(C) Summary of genome editing by the STU and STU-SR–SSA systems in rice T0 lines. Representative Sanger sequencing chromatograms of editing at the *HY::YG* reporter **(D)** and the endogenous gene loci **(E)** in obtained plants using the STU-SR–SSA system are shown.

(F) Phenotypes of the wild type (WT; left) and an *OsPDS* mutant (right) generated by the STU-SR–SSA system.

(G) Phenotypes of the WT (left) and an *OsDEP1* mutant (right) generated by the STU-SR–SSA system.

HY::YG reporter, only one of which was corrected (Figure 3B). For lines 4 and 8, the corrected *HYG* band was faint, and the *HY::YG* band was much brighter, indicating that multiple copies of the *HY::YG* reporter were present in these two lines and that most of them remained unedited or not corrected (Figure 3B). The presence of edited and/or unedited alleles of *HY::YG* and editing at the endogenous site were confirmed by Sanger sequencing (Figure 3C). For the *OsPDS*-sgRNA01 and *OsPDS*-sgRNA02 plants, the *HY::YG* reporter gene(s) were fully restored in all cases (Supplemental Figure 3). Among the eight *OsDEP1*-sgRNA01 T0 plants, five plants exhibited complete correction of the *HY::YG* reporter gene(s), and the other three plants showed partial copy correction (Supplemental Figure 3). Hence, regardless of the copy number of the *HY::YG* reporter in the regenerated plants, correction of at least one broken reporter copy seems sufficient to report robust genome editing at the endogenous loci. These data indicate that the STU-SR system is capable of

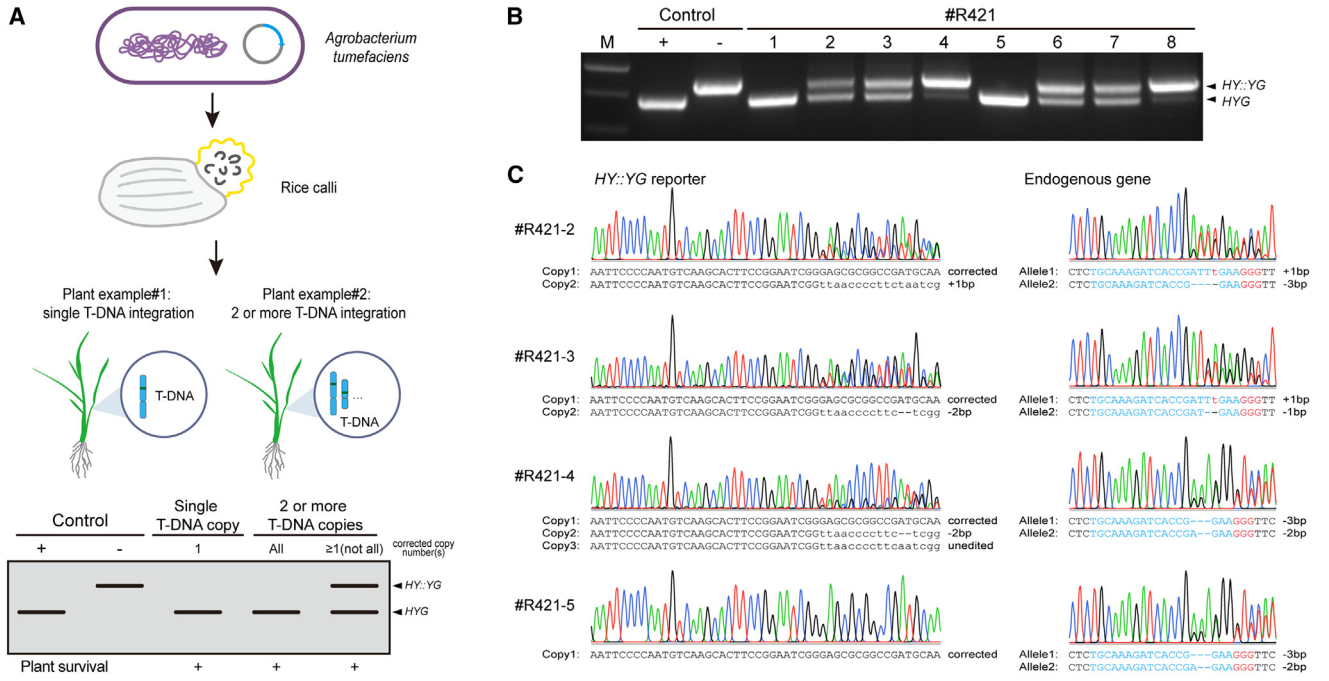


Figure 3. Enrichment of gene editing events is achieved regardless of T-DNA copy number.
(A) Schematic illustration depicting editing outcomes of the *HY::YG* reporter(s) in single or multiple T-DNA-integrated plant events.
(B) PCR detection results of the *HY::YG* reporter in the eight *OsPDS*-sgRNA04 T0 plants.
(C) Representative Sanger sequencing chromatograms and genotypes of the *OsPDS*-sgRNA04 T0 lines with editing at the *HY::YG* reporter and the endogenous gene loci by the STU-SR–SSA system.

enriching gene editing events regardless of the number of T-DNA copies, providing flexibility in its application.

Application of STU-SR–BE systems for enriching base editing events

Base editing, either via cytosine base editors (CBEs) or adenine base editors (ABEs), enables the transition of one base to another without introducing DNA DSBs. Base editing provides greater precision than targeted mutagenesis by Cas nucleases (Ren et al., 2021a; Ren et al., 2021b; Molla et al., 2021; Wu et al., 2022a). Therefore, we investigated whether the STU-SR system could also effectively enrich base editing events. Unlike the SSA enrichment strategy used for enriching CRISPR–Cas9 editing events, we developed STU-SR–CBE and STU-SR–ABE for enriching base editing events. In both cases, the surrogate reporter gene was created by strategically integrating the endogenous target site with the PAM (also known as the surrogate site) at the beginning of the *HYG* coding sequence, removing the original ATG start codon (hyg^{-ATG}) (Figure 4A). For the CBE system, when the 5′ end of the sgRNA contains "ACG," we can directly add the surrogate site in front of the hyg^{-ATG} coding sequence. If "CAC" is present at the 5′ end of the sgRNA, we add the reverse complementary sequence of the surrogate site before the hyg^{-ATG}. In both cases, ACG and CAC must be in the same reading frame as hyg^{-ATG} (Figure 4A). If needed, appropriate bases can be added between the surrogate site and the hyg^{-ATG} to ensure that they are in the same reading frame. When C-to-T editing occurs in base C of ACG or base C close to the PAM in CAC, a new ATG start codon is generated, initiating the translation of the *HYG* gene. For the ABE system, if the 5′ end of the sgRNA contains "ATA," then we can directly add the surrogate site in front of the hyg^{-ATG}. When A-to-G editing occurs in base A near the PAM in ATA, a new ATG start codon is generated, initiating translation of the *HYG* gene (Figure 4A). If the 5′ end of the sgRNA lacks the corresponding sequence (e.g., ACG or CAC for cytosine base editing or ATA for adenine base editing), one will need to adjust the protospacer sequence at the PAM distal end, allowing the presence of a 1–2 bp mismatch to form the corresponding sequence, as BEs tolerate most single or double mismatches at the distal end (Kim et al., 2019; Talas et al., 2021). Upon successful expression of active hygromycin phosphotransferase and potential base editing at the surrogate site, hygromycin-resistant plants will be obtained, which should also carry high-frequency base editing at the endogenous target site because the same sgRNA was used to edit both sites (Figure 4A).

In our demonstration, we used a PmCDA1-based CBE (Nishida et al., 2016; Tang et al., 2019) because it is a highly efficient CBE in plants with undetectable genome-wide off-target effects (Ren et al., 2021a; Randall et al., 2021). For A-to-G editing, ABE8e was chosen because of its high editing activities (Lapinaite et al., 2020; Richter et al., 2020). Also, the genome-wide off-target effects of ABE8e have been studied comprehensively in plants (Wu et al., 2022b; Sretenovic et al., 2023). As in the STU-SR–SSA system, we designed the CBE/ABE expression cassette and the hyg^{-ATG} expression cassette tail to tail for ease of cloning sgRNAs and surrogate sites into the STU-SR–BE backbone. Using forward and reverse oligos as primers, we amplified fragments from the STU-SR–BE backbones. After digestion with BsaI and AvrII (KpnI), these PCR fragments were

BE systems	Targeted gene	Construct	sgRNA	sgRNA+PAM	Tested T0 lines	*HYG* corrected T0 lines	Base edited number	Base edited ratio
STU-CBE	*OsPDS*	#R204	*OsPDS*-sgRNA02	CTTGAGCTTCAACATAAGCTTGG	19	-	1	5.3%
STU-SR–CBE		#R198			18	18	2	11.1%
STU-CBE	*OsPDS*	#R205	*OsPDS*-sgRNA07	ACTCCAGCATTGAACGGTTTGG	19	-	7	38.6%
STU-SR–CBE		#R199			18	18	16	88.9 %
STU-CBE	*OsCDC48*	#R206	*OsCDC48*-sgRNA01	ACCAGCCAGCGTCTGGCGCCGG	21	-	11	52.4%
STU-SR–CBE		#R200			20	20	16	80%
STU-CBE	*OsDEP1*	#R208	*OsDEP1*-sgRNA03	TACGAGGCGGATCGAGCTAGCGG	22	-	21	95.5%
STU-SR–CBE		#R202			23	23	23	100%
STU-CBE_SpRY	*OsPDS*	#R433	*OsPDS*-sgRNA04	TGCAAAGATCACCGATTGAAGGG	21	-	1	4.8%
STU-SR–CBE_SpRY		#R434			21	21	2	9.5%
STU-ABE_SpRY	*OsMPK6*	#R427	*OsMPK6*-sgRNA01	ACATACTCAGTCATAAAATCGGT	22	-	1	4.5%
STU-SR–ABE_SpRY		#R428			22	22	3	13.6%

Figure 4. Efficient base editing in rice by the STU-SR system.

(A) Enrichment diagram depicting the implementation of the STU-SR–BE system for base editing in rice.

(B) Schematic representation of the stepwise cloning strategy used to construct the STU-SR–BE vectors for base editing in rice. When designing the vectors, one must ensure that the newly generated ATG start codon resulting from base editing aligns with the hyg^{-ATG} coding sequence (CDS) within the same open reading frame. Otherwise, the sequence between the surrogate site and the hyg^{-ATG} CDS must be adjusted to ensure that they are in the same open reading frame. If the 5′ end of the sgRNA lacks the corresponding sequence, such as ACG or CAC in CBE or ATA in ABE, then this can be addressed by adjusting the protospacer sequence at the PAM distal end, allowing the presence of a 1–2 bp mismatch to form the corresponding sequence.

(C) Summary of base editing by the STU and STU-SR–BE systems in rice T0 lines.

(D) Examples of base-edited rice T0 lines generated by the STU-SR–BE systems at the hygromycin site and representative endogenous gene loci.

(E) Examples of base-edited rice T0 lines generated by the STU-SR–BE systems working with the SpRY variant at the hygromycin site and representative endogenous gene loci.

ligated into the STU-SR–BE backbone vectors cut with the same enzymes, resulting in the STU-SR–CBE and STU-SR–ABE expression vectors (Figure 4B). Our initial experiments evaluated the enrichment efficiency of STU-SR–CBE at four rice endogenous target sites. The T0 transgenic plants generated by STU-SR–CBE consistently exhibited correction of the surrogate reporter gene (Figure 4C). At the *OsPDS*-sgRNA02 site, the STU-SR–CBE system showed a 2.09-fold enhancement of editing efficiency compared with the CBE system (11.1% vs. 5.3%). Similarly, at the *OsPDS*-sgRNA07 site, STU-SR–CBE showed a 2.30-fold improvement relative to STU–CBE (88.9% vs. 38.6%). At the *OsCDC48*-sgRNA01 site, a 1.53-fold enhancement in editing efficiency was observed for STU-SR–CBE relative to STU–CBE (80% vs. 52.4%). At the *OsDEP1*-sgRNA03 site, the STU–CBE system achieved 95.5% editing efficiency, and STU-SR–CBE further improved the editing efficiency to 100% (Figure 4C). Sanger sequencing confirmed successful C-to-T editing at both the reporter gene and endogenous gene loci (Figure 4D; Supplemental Figure 4).

We next expanded the editing range by replacing Cas9 in the STU-SR–CBE system with SpRY, generating STU-SR–CBE_SpRY for PAM-less C-to-T base editing. When tested at the *OsPDS*-sgRNA04 site, the STU-SR–CBE_SpRY system showed a 1.98-fold increase in editing efficiency compared with STU–CBE_SpRY (9.5% vs. 4.8%) (Figure 4C and 4E; Supplemental Figure 4). Similarly, we also generated STU-SR–SpRY–ABE for enriching PAM-less A-to-G base editing. At the *OsMPK6*-sgRNA01 site, we observed a 3.02-fold enhancement in editing efficiency with STU-SR–ABE_SpRY (13.6% vs. 4.5%) (Figure 4C and 4F; Supplemental Figure 4). Together, the STU-SR–BE systems effectively enriched base editing events in every tested case, and the systems can be combined with SpRY to expand the target range for base editing.

Enhanced genome editing in *B. oleracea* with the STU-SR–SSA system

To further explore the versatility of the STU-SR system for enriching genome editing events in other plant species, we chose the dicot *B. oleracea*. The commonly used selection marker for *B. oleracea transformation* is the bialaphos resistance (*BAR*) gene. We therefore integrated endogenous gene editing sites into the *BAR* gene, flanked by homologous sequences, to construct the *BA::AR* surrogate reporter gene. The STU Cas9 expression unit and the *BA::AR* reporter expression unit were designed to be arranged tail to tail, facilitating the construction of STU-SR–SSA expression vectors that utilize the *BAR* selection marker gene (Figure 5A). When the *BA::AR* surrogate reporter gene is cleaved by a targeting sgRNA, it can be restored to a functional *BAR* gene through SSA homologous recombination. This reconstitution enables expression of the active phosphinothricin acetyltransferase enzyme. Simultaneously, the same sgRNA will target the endogenous locus to enable editing. By selecting for Basta-resistant plants, we could achieve the enrichment of events in which mutations occurred at the endogenous target gene (Figure 5A).

We selected three endogenous target loci, *BoPDS*-sgRNA01, *BoPDS*-sgRNA02, and *BoBIK*-sgRNA01, and compared their editing efficiencies in stable transgenic plants. As in rice, we compared the STU-SR–SSA system to the STU system. Transgenic T0 *B. oleracea* plants were obtained by *Agrobacterium*-mediated transformation. At the *BoPDS*-sgRNA01 site, the STU system did not generate any mutations among 60 T0 lines, whereas the STU-SR–SSA system achieved editing in 35% of 80 T0 plants. At the *BoPDS*-sgRNA02 site, the STU-SR–SSA system exhibited a 3.34-fold improvement in editing efficiency compared with the STU system (5% vs. 16.7%). At the *BoBIK*-sgRNA01 site, the STU-SR–SSA system demonstrated a remarkable 48.4% increase in editing efficiency, reaching nearly 80% (Figure 5B). Hence, the STU-SR–SSA system effectively enriched editing events at all three endogenous target sites (Figure 5B). Phenotypic analysis of the regenerated T0 plants revealed that the STU-SR–SSA system generated biallelic and homozygous mutations much more frequently than did the STU system for both *BoPDS* (Figure 5C) and *BoBIK* (Figure 5D), producing loss-of-function phenotypes such as photobleaching and dwarfism, respectively, as reported previously (Ma et al., 2019). Sanger sequencing confirmed the restoration of the *BA::AR* surrogate reporter gene (Figure 5E) and demonstrated successful editing at the endogenous gene loci (Figure 5F). In summary, experiments in *B. oleracea* demonstrated the effectiveness of the STU-SR–SSA system for enriching gene editing events in dicotyledonous plants.

DISCUSSION

CRISPR–Cas-based genome editing systems have been rapidly adopted by plant researchers for investigation of gene function and development of improved crops. As DNA targeting and editing are programmed by gRNAs, it is inevitable that editing efficiency at different target sites may vary significantly. In addition, chromatin status and epigenetic features also play a role in influencing editing outcomes (Liu et al., 2019; Weiss et al., 2022). Currently, we do not fully understand how each CRISPR–Cas system functions optimally in plants or the rules for designing the most efficient gRNAs for each application. Furthermore, target sites in base editing or prime editing experiments may be pre-defined, and there is a lack of flexibility for altering the protospacer sequences to boost genome editing activity. Given these circumstances, it is immensely significant if we can enrich gene editing events. It will help accelerate the pace of scientific investigation and plant breeding initiatives, enabling researchers to obtain edited plants more rapidly and efficiently, thereby saving valuable time and resources. In the context of complex plant genomes, in which specific editing events can be exceedingly rare and challenging to obtain, enriching these events substantially increases the likelihood of achieving the desired genetic modifications, particularly for intricate editing tasks.

Previously, surrogate reporter systems were developed to enrich genome editing outcomes for targeted mutagenesis by CRISPR–Cas9 (Tian et al., 2022), base editing (Xu et al., 2020b), and prime editing (Xu et al., 2020a; Li et al., 2020). However, these surrogate reporter systems each rely on a pre-defined sgRNA to restore the broken reporter to a functional one. These systems can therefore enrich transgenic lines with high levels of Cas expression but cannot necessarily enrich lines with high sgRNA expression and activity for the endogenous target sites (Figure 1B). We reasoned that an ideal and efficient surrogate reporter system would enable direct selection of edited lines based on the

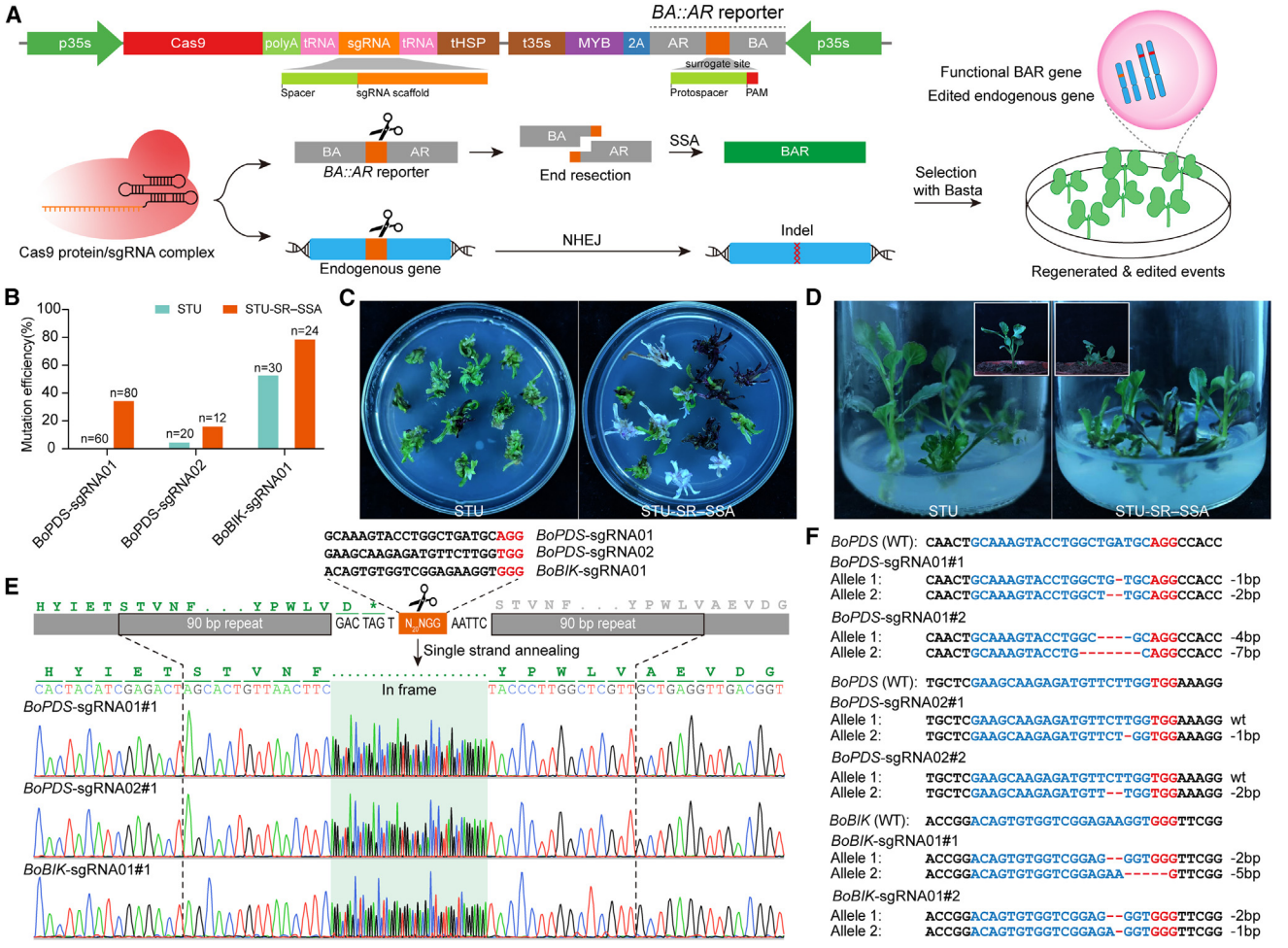


Figure 5. Efficient gene editing in *Brassica oleracea* by the STU-SR system.

(A) Schematic representation of the STU-SR–SSA vector and its implementation for achieving enrichment of gene editing events in *B. oleracea*.
(B) Comparison of editing efficiencies at three endogenous gene loci using STU and STU-SR–SSA systems.
(C) Representative images of *BoPDS* gene editing using STU and STU-SR–SSA systems.
(D) Representative images of *BoBIK* gene editing using STU and STU-SR–SSA systems.
(E) Representative Sanger sequencing chromatograms of T0 lines with editing at the *BA::AR* reporter locus.
(F) Genotypes of T0 lines with gene editing by the STU-SR–SSA system in *B. oleracea*.

sgRNAs targeting the genes of interest (Figure 1C). However, at first glance, it is very challenging to develop such surrogate reporter systems. After all, the sequences of the reporter genes are pre-defined, but the target sequences vary from experiment to experiment, depending on the genes of interest. We employed different strategies to solve this problem. To develop surrogate reporter systems for CRISPR–Cas9, we used SSA that relies on the tandem repeat sequences flanking the DNA DSB, as SSA is an efficient DSB repair mechanism conserved in a variety of organisms, including yeast (Paques and Haber, 1999), humans (Bhargava et al., 2016), and plants (Puchta, 2005; Zhang et al., 2013). Indeed, our STU-SR–SSA reporter systems based on different selection markers worked well, significantly enriching editing events in rice (Figure 2) and *B. oleracea* (Figure 5). Furthermore, the STU-SR–SSA reporter systems worked robustly, regardless of the T-DNA copy number (Figure 3). These data suggest that SSA-based surrogate systems are widely applicable in plants. They should be compatible with biolistic transformation methods, which often generate transgenic plants with multiple copies of the transgene. Although we demonstrated the STU-SR–SSA systems with CRISPR–Cas9, the same SSA principle can be used to develop surrogate reporter systems for other CRISPR systems such as CRISPR–Cas12a (Tang et al., 2017; Zhong et al., 2018), CRISPR–Cas12b (Ming et al., 2020), and compact CRISPR–Cas12j2 (Liu et al., 2022).

Compared with CRISPR–Cas-mediated targeted mutagenesis, base editing and prime editing are more precise genome editing tools for installing base-precision changes in genomes (Anzalone et al., 2020). However, these tools often suffer from low editing efficiency, making their use in plant applications riskier and more laborious (Tang et al., 2020; Molla et al., 2021). In this study, we developed STU-SR–CBE and STU-SR–ABE systems to enrich C-to-T and A-to-G base editing events, respectively. In our strategy, the surrogate site (encompassing the protospacer and PAM) was added to the 5′ end of the reporter gene with its original start codon deleted. The start-codon-less reporter gene is restored

when a start codon is created via base editing (Figure 4A). Our data showed that such STU-SR–BE systems significantly enriched base editing events, and the systems are compatible with Cas9 variants such as SpRY (Figure 4C). It is of note that the targets "C" for the CBE reporter and "A" for the ABE reporter reside at the 2nd and 3rd positions of the protospacer, respectively (Figure 4A), which are not necessarily within the optimal base editing windows for all BEs. However, the fact that base editing at these positions occurred with the BEs tested in this study suggests that STU-SR–BE strategies could work for many, if not all, BEs.

Prime editing in plants has been under constant improvement. For example, prime editing has become a relatively reliable tool for precise genome editing in monocots such as rice thanks to the innovative strategies employed for these improvements (Tang et al., 2020; Lin et al., 2021; Jiang et al., 2022; Xu et al., 2022; Gupta et al., 2023; Qiao et al., 2023). However, little success has been reported for prime editing in dicot plants (Lu et al., 2021). Owing to its low editing efficiency, prime editing can greatly benefit from surrogate-reporter-based enrichment systems, as demonstrated recently (Xu et al., 2020a; Li et al., 2020; Li et al., 2022). Because the targeted DNA changes in prime editing vary from site to site and the editing is more complex than base editing, a different strategy is needed to develop authentic surrogate reporters that install the surrogate site into the reporter gene. We envision that the surrogate site can be added to the start-codon-less reporter gene, as is the case for base editing reporter systems. A specific prime-editing gRNA needs to be designed to create a start codon, likely around or downstream of the Cas9 cleavage site (or 3 bp upstream of the PAM), as that region is most amenable to prime editing (Sretenovic and Qi, 2022). This strategy, in principle, should work and is worth testing. Such surrogate-reporter-based prime editing systems may enable reliable prime editing in dicot plants in the future.

In summary, we developed multiple STU-SR systems for CRISPR–Cas-mediated targeted mutagenesis, cytosine base editing, and adenine base editing in plants. The successful application of the STU-SR system in rice (a monocot) and *B. oleracea* (a dicot) demonstrates its versatility and effectiveness, indicating its potential for application across a broad spectrum of plant species. Continued improvement and expansion of this technology are expected to enable a wide range of genome editing applications in plants. The successful STU-SR strategies reported here may serve as a valuable reference and inspiration for the development of other genome editing technologies to augment plant research and applications.

METHODS

Vector construction

The STU-SR systems used in this study were generated using the following plasmids: pTX172 (Addgene #89259), pGEL031 (Addgene #137900), pGEL035 (Addgene #137903), pYPQ166-SpRY (Addgene #161520), and pYPQ262B-ABE8e (Addgene #161524). The rice STU-SR–SSA system was created by assembling a PCR-amplified backbone fragment into pTX172 (Zhou et al., 2022) via Gibson Assembly, removing the original *HYG* expression cassette. Subsequently, the *HY::YG* surrogate reporter gene was obtained through overlap PCR. Together with the 35S promoter and 35S terminator, it was assembled via Gibson Assembly into the vector behind the STU-Cas9 expression cassette, resulting in the construction of the STU-SR–SSA backbone vector pGEL901 (Addgene #218546). Similarly, the STU-SR–SSA_SpRY backbone vector pGEL902 (Addgene #218547) was constructed by replacing the Cas9 and *HYG* expression units of pGEL031 with the PCR-amplified SpRY from pYPQ166-SpRY and the *HY::YG* surrogate reporter gene expression unit. For the STU-SR–CBE system, the *HYG* expression cassette in pGEL035 was removed. Subsequently, the hyg^{-ATG} surrogate reporter unit was cloned at the SacI site of pGEL035 to produce the STU-SR–CBE backbone pGEL903 (Addgene #218548). The PCR-amplified SpRY fragment from pYPQ166-SpRY was then used to replace the Cas9 segment, resulting in the generation of the STU-SR–CBE_SpRY backbone vector pGEL904 (Addgene #218549). Similarly, the STU-SR–ABE_SpRY backbone vector pGEL905 (Addgene #218550) was constructed by replacing the CBE and *HYG* expression units of pGEL035 with the PCR-amplified SpRY-ABE8e from pYPQ262B-ABE8e and the hyg^{-ATG} surrogate reporter unit. The *ZmUbi1* promoter and *HY::YG* surrogate reporter gene of the rice STU-SR–SSA system were replaced with the 35S promoter and *BA:AR-2A-MYB* surrogate reporter gene, resulting in construction of the *B. oleracea* STU-SR–SSA backbone vector pGEL906 (Addgene #218551). Construction of the STU-SR system expression vector, as shown in Figures 2B and 4B, begins with specific cleavage of the vector backbone using restriction endonucleases to generate sticky ends. Subsequently, forward and reverse oligonucleotides are designed to incorporate flanking restriction enzyme sites, sgRNA sequences, and surrogate sites. These oligonucleotides are amplified via PCR to obtain insertion fragments containing the required sgRNA, the terminator of the Cas9 expression unit, and part of the surrogate reporter unit (including corresponding surrogate sites). The PCR products are then digested with the corresponding restriction endonucleases to create ends that are complementary to the sticky ends of the vector backbone. Ligation of these digested fragments into the vector backbone via T4 DNA ligase facilitates assembly of the expression vector by the pairing of compatible ends. The primer sequences are listed in Supplemental Table 1. All vectors used in this study are available from Addgene.

Rice stable transformation

The *Oryza sativa* japonica cultivar Nipponbare was used in this study. Rice stable transformation was performed following previously published protocols (Zhou et al., 2017; Zheng et al., 2023). Binary vectors were transformed into *Agrobacterium* strain EHA105 using the freeze-thaw method. Rice seeds were sterilized and cultured on N6-D solid medium in the light at 32°C for 2–3 weeks. Rice calli were immersed in the *Agrobacterium* suspension, dried on filter paper, and co-cultured on solid medium at 25°C in the dark for 3 days. Infected calli were washed and transferred to N6D-S screening medium at 32°C for 2 weeks. Actively growing calli were moved to RE-III regenerative medium at 28°C with a 16 h light/8 h dark cycle for 3–4 weeks. Regenerated seedlings were transferred to rooting medium for 2–3 weeks. Transgenic rice plants were grown in a growth chamber at 28°C under a 16 h light/8 h dark cycle.

B. oleracea stable transformation

The *B. oleracea* line "159," previously developed in our laboratory, served as the plant material. *Agrobacterium*-mediated transformation was performed as described previously (Ma et al., 2019). In brief, hypocotyls from 7- to 10-day-old seedlings were chosen as the target explants and were pre-cultured on callus initiation medium for 2 days. The pre-incubated hypocotyls were then immersed in an *Agrobacterium*-infection buffer and co-cultivated in the dark at 25°C for 48 h. After co-cultivation, the explants were transferred to medium optimized for callus and shoot induction. Once the regenerating shoots reached a height of 1–2 cm, they were carefully moved to a rooting medium to obtain transgenic T0 plants. Transgenic plants were transplanted into soil.

Mutagenesis analysis

Genomic DNA was extracted from transgenic plants using the cetyltrimethylammonium bromide method as reported previously (Zhou et al., 2022; Zheng et al., 2023), and PCR amplification of the target gene was performed with the specific primers listed in Supplemental Table 1. Both

single-strand conformation polymorphism analysis (Zheng et al., 2016) and Sanger sequencing (Zhou et al., 2019) were used to detect mutations. The genotypes of T0 mutant lines were analyzed using CRISPR-GE DSDecodeM software (Xie et al., 2017).

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

Y.Z. proposed the project and designed the experiments. X.T., Q.R., and R.Z. constructed all the plasmids. X.T., Q.R., L.L., Q.H., and X.Z. performed the rice stable transformation. X.T., Q.R., and R.Z. analyzed the T0 rice plants. X.Y. and H.S. performed the *B. oleracea* stable transformation and analyzed the T0 *B. oleracea* plants. Y.Z., Y.Q., X.T., and H.S. analyzed the data and wrote the manuscript with input from other authors. All authors read and approved the final version of the manuscript.

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