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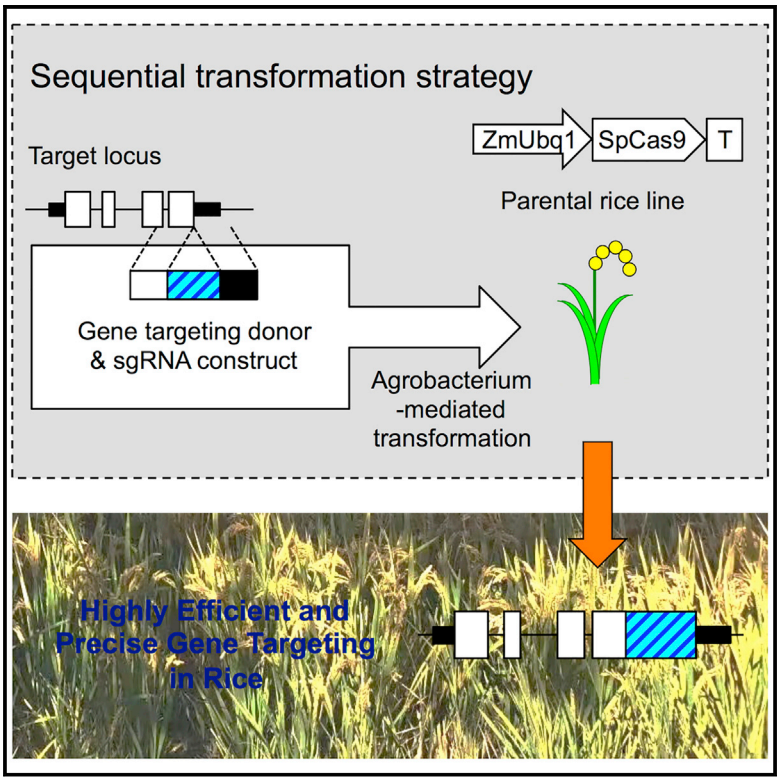
Precise and heritable gene targeting in rice using a sequential transformation strategy

Graphical abstract



Highlights

- An efficient and precise CRISPR-Cas9-mediated gene targeting method in rice
- Precise *GFP* knockin to the endogenous *OsFTL1* and *OsROS1a* loci are achieved
- The sequential transformation strategy can be applied to a wide variety of plants
- Epigenetic DNA methylation modifications at target loci are not altered by GT

Authors

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In brief

Zhang et al. establish a procedure for the precise replacement or knocking in of a specific sequence at an endogenous locus in rice, one of most important crops in the world. Stable rice plants with GFP knockin at the endogenous OsROS1a and OsFTL1 loci are generated with high efficiency.

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Precise and heritable gene targeting in rice using a sequential transformation strategy

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MOTIVATION Gene targeting of specific endogenous loci is difficult in spermatophytes such as rice due to low homology-directed repair (HDR) efficiency and difficulty in deriving donor templates. To address this, we adapted a method developed in *Arabidopsis* that uses a CRISPR-Cas9-mediated sequential transformation strategy to insert modules at specific target locus in the plant genome with high efficiency and accuracy. In this study, rice parental lines were generated and sequential transformation strategies were used to demonstrate highly efficient and precise knockin of *GFP* at two loci. Compared with other SSN-mediated GT approaches, this sequential transformation strategy does not rely on selection markers used to improve screening efficiency for antibiotic or herbicide resistance genes at the target loci. Moreover, our results strongly suggest that not only the primary DNA sequence, but also epigenetic modifications, especially DNA methylation at target loci, are unaltered. Thus, our GT technology will be applied to molecular research and breeding of rice and other crop species.

SUMMARY

Gene targeting (GT) is a powerful tool for modifying endogenous genomic sequences of interest, such as sequence replacement and gene knockin. Although the efficiency of GT is extremely low in higher plants, engineered sequence-specific nucleases (SSNs)-mediated double-strand breaks (DSBs) can improve GT frequency. We recently reported a CRISPR-Cas9-mediated approach for heritable GT in *Arabidopsis*, called the "sequential transformation" strategy. For efficient establishment of GT via the sequential transformation method, strong Cas9 activity and robust DSBs are required in the plant cells being infected with *Agrobacterium* carrying sgRNA and donor DNA. Accordingly, we generated two independent parental lines with maize *Ubiquitin 1* promoter-driven Cas9 and established sequential transformation-mediated GT in the Japonica rice cultivar *Oryza sativa* Nipponbare. We achieved precise *GFP* knockin into the endogenous *OsFTL1* and *OsROS1a* loci. We believe that our GT technology could be widely utilized in rice research and breeding applications.

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INTRODUCTION

Site-specific genome modification by engineered sequence-specific nucleases (SSNs) is a powerful tool for generating targeted mutations. In recent years, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has been widely used to generate site-specific double-stranded breaks (DSBs), which are recognized in a sequence-specific manner by the single guide RNA (sgRNA), for genome editing in numerous organisms, including plants.[1–5] The generated DSBs are repaired by either error-prone non-homologous end-joining (NHEJ) or error-free homology-directed repair (HDR) if a precise repair donor template is provided.[6] Repair of DSBs via the error-prone NHEJ pathway leads to random mutations in the target sites. On the other hand, HDR-mediated gene targeting (GT) generates precise genome modifications, such as sequence replacement and knockin. GT is a powerful tool for genome engineering and is widely applied in many organisms, including *Drosophila* and animals.[7–9] Nevertheless, GT is still a challenging task, especially in higher plants, because of the extremely low frequency of homologous recombination.[10]

GT in higher plants was first reported in tobacco somatic leaf tissues.[11] A heritable GT method relying on positive-negative selection markers was later developed in rice,[12] but the method is still complicated and difficult to use.[13–16] It has been reported that SSNs can facilitate the efficiency of GT in many systems, including human stem cells.[7,17] The introduction of DSBs also increases the frequency of homologous recombination in plants.[18,19] Success in HDR-mediated GT using SSNs was recently reported in some plant species, including *Arabidopsis* and rice.[20,21] The majority of reported GT events relied on selection markers for antibiotic or herbicide resistance genes at the target loci to improve screening efficiency. A few reported events did not use selection markers, but GT frequencies were low.[21–26]

Previously, we reported a sequential transformation method for CRISPR-Cas9-mediated efficient GT in *Arabidopsis*.[27] Our sequential transformation method is to deliver an HDR donor construct, which also harbors an sgRNA targeting the gene of interest, into parental transgenic plants that stably express Cas9. Most of the GT reports in higher plants used an all-in-one system that contained an SSN to generate DSB at the target locus and an HDR donor sequence. While SSNs-mediated DSB efficiencies vary among independent transformants in the all-in-one system, high Cas9 activity, reflected as DSB efficiency, is secured in the sequential transformation method to confer consistently high GT efficiencies.

A *DNA ligase 4* (*Lig4*) mutant background- and geminivirus replicon-based GT via sequential transformation in rice has been reported.[28,29] *Lig4* is required for efficient repair for non-homologous end-joining, and its loss-of-function mutant increases the HDR frequency and unwanted spontaneous mutations.[30,31] Furthermore, the geminivirus replicon has a limited host range, and it can be difficult to construct viral vectors. In the present study, we show that the sequential transformation strategy can be used to establish GT in rice. Our sequential transformation strategy is expected to facilitate GT in higher plant species, including crops.

RESULTS

Generation and characterization of parental rice lines

To establish a sequential transformation method for GT in rice (Figure 1A), the generation of highly efficient parental lines is necessary. The maize *Ubiquitin 1* (ZmUbq1) promoter is a strong constitutive promoter in monocot plants[32,33] and has been used for a wide range of studies, including GT. We made a construct to express the optimized coding sequence of *Streptococcus pyogenes* Cas9 (SpCas9; here after Cas9) (Figure 1B). For the parental line construct, an sgRNA was designed at an intergenic region between LOC_Os01g16450 and LOC_Os01g16460, in which two genes are located in the tail-to-tail direction. Theoretically, the possibility that mutations at this intergenic region will affect gene expression and genomic stability may be very low.

We obtained 26 independent T0 transgenic rice lines. For characterization, the Cas9 expression and mutation ratio of the target locus were determined using callus tissues and leaf tissue of the regenerated plants, respectively (Figure 1C). Interestingly, Cas9 expression levels were not correlated with the target mutation ratio in the ZmUbq1 pro:Cas9 rice lines (Figure 1D). Based on the quantitative Chop-PCR (qChop-PCR) results (Figure 1C), further analysis focused on the possible candidates of parental transgenic rice lines. The mutation of the target locus was also detected by restriction-length polymorphism (RFLP) for the top nine lines of mutants (Figure 1E). The results of RFLP are reproducible with qChop-PCR. The top nine lines of ZmUbq1 pro:-Cas9 showed almost 100% mutation (Figure 1E). To determine the mutation patterns, the PCR products without restriction enzyme digestion were subcloned into a cloning vector followed by Sanger sequencing analysis for 10 to 16 independent clones. The biallelic mutations were mainly detected in ZmUbq1 pro:-Cas9 T0 lines (Table 1). The biallelic mutants would have been established in the primary single-cell or early stage of the transformant, which was *Agrobacterium* infected,[34] but not in the later vegetative growth stage. These results indicate that higher Cas9-mediated DSB generation activity in callus tissue must be one of the key factors for generating and obtaining biallelic mutations in rice.

The top four mutation ratio T0 lines (#172, #178, #167, #180) were selected, and these progeny T1 generation rice individual plants were analyzed to confirm whether the detected mutation in T0 rice plants was heritable. The RFLP genotyping results of ZmUbq1 pro:Cas9 T1 plants showed almost 100% mutation (Figure 1F). Furthermore, several plants that were homozygous or biallelic mutants without the Cas9 transgene were identified from these T1 populations (Figure 1F). The detailed sequencing analysis of the mutations in the T1 plants indicated that the majority of the mutations were biallelic and heritable (Table 2). Surprisingly, a new mutation was detected in #178 T1, even though the sequencing result of T0 indicated biallelic mutation (Tables 1 and 2). The molecular mechanism is unknown, but might be due to the presence of an undetected, unexpected mutation in T0 or unwanted retargeting by Cas9. The sequencing results indicated that ZmUbq1 promoter-driven Cas9 would have strong DSB activity at a very early stage of transformation and the DSB could occur in the primary single-cell or early stage, just after infection by *Agrobacterium*. Altogether, these results suggest that the

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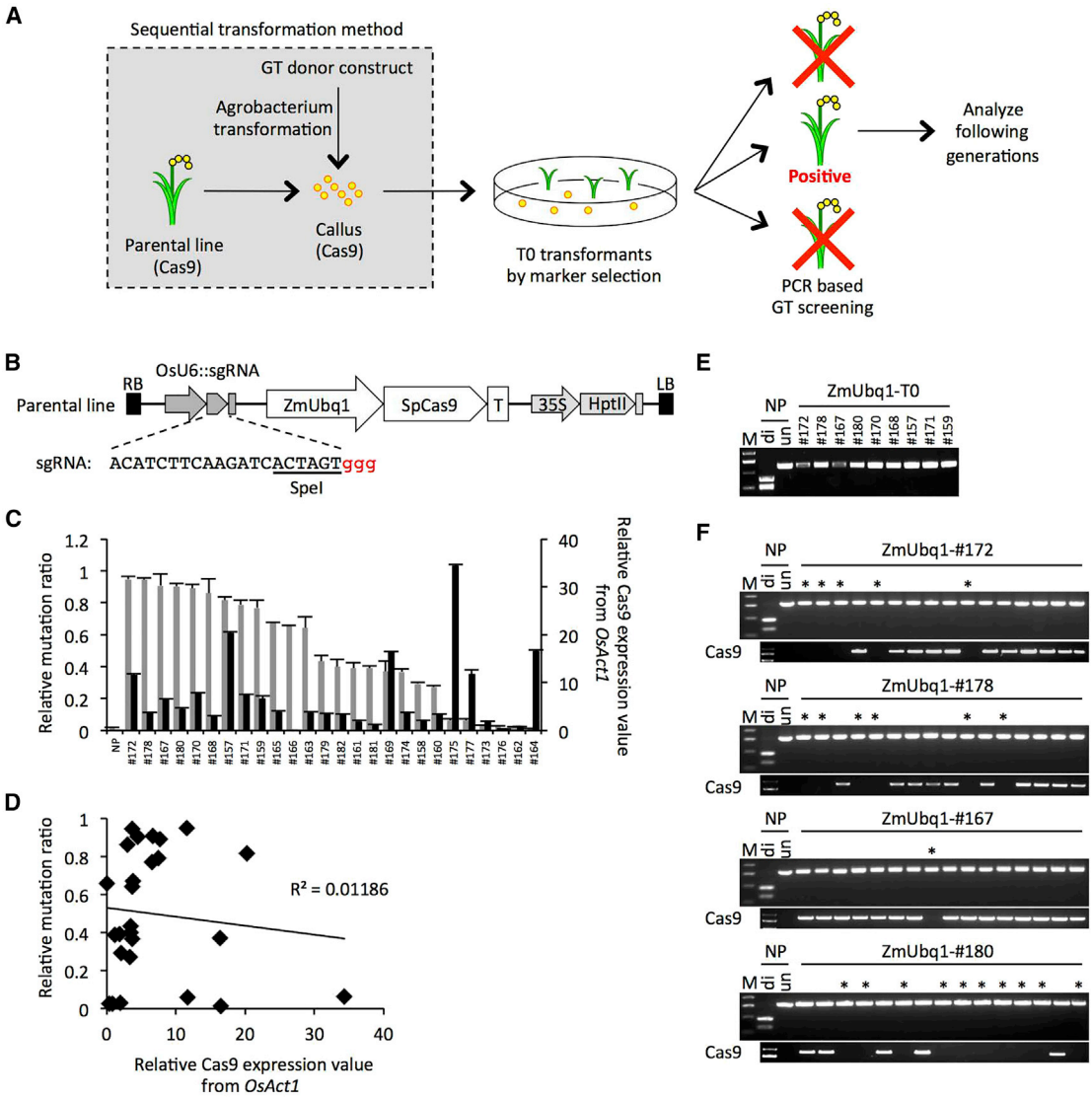

Figure 1. Generation of rice parental lines

(A) Outline for the sequential transformation method in rice. GT donor constructs with sgRNAs targeting genes of interest and containing a selection marker were transformed into the parental line, which is already transgenic for an incidental intergenic region-targeting sgRNA and for Cas9 driven by the ZmUbq1 promoter. T0 transgenic lines were selected with Basta, and individual T0 plants were analyzed. The positive lines were used for further experiments.

(B) Rice parental line ZmUbq1 pro:Cas9 construct. Schematic represents the transfer DNA construct for the rice parental line, which harbors Cas9 driven by the ZmUbq1 promoter, an sgRNA driven by the OsU6 promoter, and the hygromycin selection marker *HptII* gene driven by the CaMV 35S promoter.

(C) Mutation efficiency and Cas9 expression in transgenic T0 rice. The mutation ratio was determined by qChop-PCR (gray bar). The relative Cas9 expression value was calculated from *OsAct1* (black bar). The error bars indicate standard deviations (n = 3).

(D) Relationship between Cas9 expression and mutation among ZmUbq1 transgenic T0 lines. The Cas9 expression level and mutation ratio in the 26 independent ZmUbq1 pro:Cas9 transgenic T0 lines were plotted. The mutual fund is shown by the coefficient determination (R^2).

(E) Mutation of the target locus genotype in selected T0 transgenic rice plants.

(F) Mutation of the target locus and Cas9 genotype in T1 transgenic rice plants. The mutation genotype was determined by RFLP. The PCR products were digested with SpeI. Asterisks indicate Cas9 transgene-free homozygous or biallelic mutant rice plants. NP, nontransgenic Nipponbare as a control; di, SpeI digested; un, SpeI undigested controls.

lines with constitutively strong ZmUbq1 promoter-driven Cas9 activity can be candidate parental lines for GT via sequential transformation. We used two ZmUbq1 pro:Cas9 lines (#167 and #180) as the parental lines for sequential transformation, due to their mutation pattern and availability of seeds.

Heritable *GFP* knockin GT at the *OsFTL1* locus

We made an HDR donor, which harbors 800 base pair (bp) flanking sequences as homology arms, with an sgRNA construct to generate *OsFTL1-GFP* knockin GT rice (Figure 2A and S1). The GT donor construct was transformed via

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Table 1. Sequencing result of mutation at the target locus in T0 parental line transgenic rice plants

Line name	Mutation pattern	Read numbers	Total reads	Possible mutation type
#172	A deletion	5	13	biallelic
	3 bp deletion	8		
#178	A insertion	5	10	biallelic
	A deletion	5		
#167	T insertion	3	13	biallelic (chimeric)
	4 bp deletion	10		
#180	3 bp deletion	10	10	biallelic
#170	3 bp deletion	7	14	biallelic
	4 bp deletion	7		
#168	A deletion	9	15	biallelic
	3 bp deletion	6		
#157	A deletion	2	14	chimeric
	T deletion	6		
	4 bp deletion	6		
#171	A deletion	10	16	biallelic
	6 bp deletion	6		
#159	A insertion	7	16	biallelic
	T insertion	9		

Table 2. Sequencing result of mutation at the target locus in individual T1 parental line transgenic rice plants

Line name	Mutation pattern	Individual plant numbers	Total plant numbers	Original mutation type
#172	A deletion	4	16	biallelic
	3 bp deletion	2		
	hetero	10		
#178	A insertion	5	16	chimeric
	A deletion	3		
	2 bp deletion	2		
	chimeric	6		
#167	T insertion	4	16	biallelic
	4 bp deletion	3		
	hetero	9		
#180	3 bp deletion	15	15	biallelic

Agrobacterium into callus tissues of the two parental ZmUbq1 pro:Cas9 lines (#167 and #180), which were grown on hygromycin-containing media. T0 transgenic lines from this second round of transformation were selected using the Basta resistance gene. GT events were analyzed by PCR for each of the independent T0 transgenic rice plants. We used two primer sets for PCR-based genotyping. One set had a primer within the knockin *GFP* sequence and only amplified the knockin allele (GT-specific primers), while the other primer set had a primer within the homology arm sequence and amplified both the endogenous and knockin alleles (external primers) (Figure S1A). According to our work in *Arabidopsis*, the GT-specific primer set detects some false-positive signals, which likely come from nonheritable GT events in minor somatic tissues that were not detected by the external primer set.[27,35,36] *OsFTL1-GFP* GT-positive T0 rice plants were obtained from the parental lines #167 and #180, which yielded seven and two plants, respectively (Figure 2B and Table 3). All GT-positive T0 rice plants could be confirmed by Southern blot analysis using SpeI and HpaI restriction enzymes (Figures 2B and S1A). The sgRNA applied to generate *OsFTL1-GFP* also targets the 5′ homology arm of the donor construct, such that silent mutations were introduced at the sgRNA recognition site in the HDR donor sequence to prevent sgRNA binding and the generation of DSB and mutations following precise knockin (Figure S1A).[21] Sanger sequencing of PCR products confirmed that all GT events were precise and accurate, including intended silent mutations (Figure 2C). The GT efficiencies for *OsFTL1-GFP* in T0 rice plants were 8.9% and 3.03% for the parental lines #167 and #180, respectively (Table 3).

All precise *OsFTL1-GFP* GT events were stably inherited and showed Mendelian segregation in the T1 generation (Figure 2D). Furthermore, we obtained one donor transgene-free *OsFTL1-GFP* T1 rice plant from line #30, and another *OsFTL1-GFP* T1 rice plant free from all transgenes, including the Cas9, from the first transformation and the donor from the second transformation, from line #38 (Figure 2D). It has been reported that GT frequencies appear to be enhanced when both donor and target genes are located near the same chromosome in *Arabidopsis* and barley.[10,37] However, our results suggest that the tight physical linkage between the GT locus and donor transgene might not be necessary for efficient GT establishment in rice. This result is also consistent with previous reports.[21]

Heritable *GFP* knockin GT at the *OsROS1a* locus

Next, to investigate the broad utility of our sequential transformation method-mediated GT, we attempted to generate an in-frame *GFP* knockin at the 3′ end of *OsROS1a*, a DNA glycosylase/lyase gene for active DNA demethylation. We designed a donor construct for *OsROS1a-GFP* with a specific sgRNA and 1K bp homology arms for HDR-mediated GT (Figure 3A). The donor transfer DNA construct was transformed into the parental line #180. We obtained one *OsROS1a-GFP* GT-positive plant from 33 independent Basta-resistant T0 transgenic rice plants (Figure 3B and, Table 3). This GT event was precise and heritable, segregating into homozygous and heterozygous plants in the T1 generation (Figure 3C). Furthermore, a donor transgene-free homozygous and heterozygous *OsROS1a-GFP* GT T1 plant was obtained (Figure 3C). Taken together, our sequential transformation strategy can be a powerful tool for GT in crop plants.

The loss-of-function *osros1a* mutant is embryonic lethal,[38] and weak *osros1a* mutant alleles result in a thickened aleurone layer in rice grains of the rice cultivar Zhonghua 11.[39] We found that *OsROS1a-GFP* expression in homozygous GT T1 generation plants was comparable to endogenous *OsROS1a* expression in Nipponbare (Figure 3D). Furthermore, the size of the aleurone layer in rice grains was comparable between the nontransgenic Nipponbare, parental lines, and *OsROS1a-GFP* homozygous GT lines (Figure 3E). These results indicate that the *GFP* knockin

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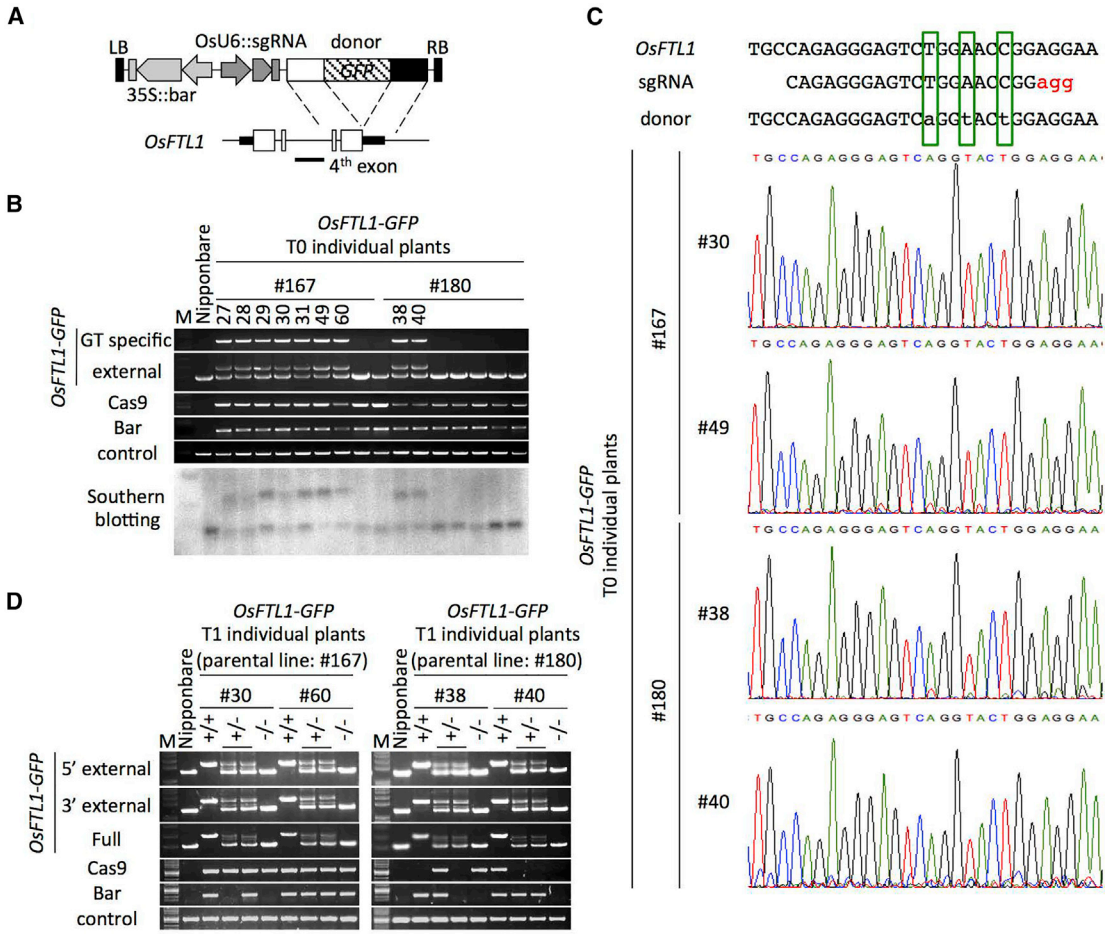

Figure 2. *GFP* knockin into the *OsFTL1* locus

(A) Schematic of the *OsFTL1-GFP* donor construct and endogenous target locus. The donor construct contains an in-frame *GFP* sequence with endogenous *OsFTL1* harboring 800 bp homology arms (Figure S1A). Horizontal line represents the probe for Southern blotting (Figure S1A).

(B and D) PCR and Southern blot hybridization genotyping of *OsFTL1-GFP* T0 (B) and T1 (D).

(C) Sequence confirmation of the precise *GFP* knockin for T0 *OsFTL1-GFP*. Silent mutations are indicated by green rectangles.

into the endogenous locus does not interfere with *OsROS1a* expression and its function.

Epigenetic effects at the GT target locus *OsFTL1*

GT-associated alterations in epigenetic status were analyzed in *Arabidopsis*,[23,27,40] but have not been investigated in other plant species, including rice. To examine whether GT affects epigenetic modifications at the GT target locus, the DNA methylation status was analyzed in *OsFTL1-GFP* knockin GT rice. We performed individual bisulfite sequencing of the 5′ and 3′ homology arm regions in Nipponbare and all nine independent *OsFTL1-GFP* knockin T0 plants. We did not observe substantial changes in cytosine methylation at the 5′ and 3′ homology arm regions (Figure 4A). Moreover, we examined whether *de novo* DNA methylation is established at the knockin *GFP* sequence. We performed individual bisulfite sequencing for transgene-free *OsFTL1-GFP* knockin T1 plants, which are shown in Figure 2D. We also did not observe *de novo* methylation at the knockin *GFP* sequence (Figure 4B). Taken together, these results suggest that epigenetic status, specifically DNA methylation, is not altered or established *de novo* by GT in rice or *Arabidopsis*.[23,27,40]

DISCUSSION

Interestingly, while some papers report a positive correlation between Cas9 expression and mutation frequency,[41] in the T0 ZmUbq1 pro:Cas9 rice lines in this study, Cas9 expression did not correlate with target mutation rate. For characterization, we analyzed the expression level of Cas9 using callus tissue and the mutation ratio of the target locus in leaf tissue of regenerated rice plants, respectively. This may be one reason why Cas9 expression levels do not correlate with the mutation ratio of the target locus in this study. Furthermore, the DSB efficiency of Cas9 may depend not only on the expression levels, but also translation, sgRNA expression, Cas9 protein and sgRNA complex formation, and so on. However, the strong DSB activity of Cas9 in the callus tissue in later generations is probably the most critical factor for the efficient establishment of GTs by the

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Table 3. Knockin GT efficiencies in rice

Construct	Parental line	T0 transgenic lines: Transformant	GT positive	GT efficiency (%)
OsFTL1-GFP	#167	78	7	8.9
	#180	66	2	3.03
OsROS1a-GFP	#180	33	1	3.03

GT efficiency was calculated based on the number of individual T0 transformant populations examined.

sequential transformation strategy. If a rice plant is regenerated from one or a few cells with high mutation frequency, these plants are expected to be useful parental lines with high Cas9 activity for sequential transformation.

We have reported efficient and heritable GT in *Arabidopsis* via the sequential transformation method.[27] Only DD45 pro:Cas9, which stably expresses the Cas9 nuclease in egg cells and the early embryo in parental lines, yielded heritable GT, indicating that high DSB efficiency in *Agrobacterium*-infected cells may be the most important factor for GT establishment.[35] *Agrobacterium* releases a large amount of single-stranded transfer DNA (ssT-DNA) into the infected cells, which are germline cells, as in the case of the flower dipping method for *Arabidopsis*.[42,43] We proposed that the released ssT-DNA could be the most likely candidate of the donor molecule for HDR-mediated GT.[20,35] Hence, GT could be established immediately after transformation when *Agrobacterium* infects Cas9-expressing cells. Based on this idea, we hypothesized that the DD45 ortholog promoter from rice would not be feasible for the establishment of heritable GT in rice because of the difference in the *Agrobacterium*-mediated transformation method. Thus, we used a strong constitutive maize *Ubiquitin 1* (ZmUbq1) promoter to drive Cas9 in this research instead of germline-specific promoters. Furthermore, previous studies have used and successfully obtained SSN-mediated heritable GT in rice by the all-in-one method.[20] Here, we achieved precise knockin generation of *OsFTL1-GFP* and *OsROS1a-GFP* by using a sequential transformation method. Accordingly, the ZmUbq1 promoter enables heritable GT establishment in rice.

Based on our work and other previous studies, the sequential transformation method can yield higher GT efficiency than the all-in-one method.[23,27–29] The higher GT efficiency can be explained by the fact that the plants with higher DSB efficiency, determined by Cas9 activity, were selected as the parental line for sequential transformation. However, Cas9 activity is variable among independent transgenic lines when the all-in-one method is applied. This could be one of the key reasons that the sequential transformation shows higher GT efficiency. Theoretically, the sequential transformation method would be applicable to other crop plants. However, parental lines with high Cas9 activity are necessary for sequential transformation, which is time-consuming and limits its application in diverse genetic backgrounds.

The epigenetic effects at the target loci caused by GT events have rarely been analyzed. Reduction of DNA methylation was reported with ZFN-mediated GT at the endogenous *PPOX* locus

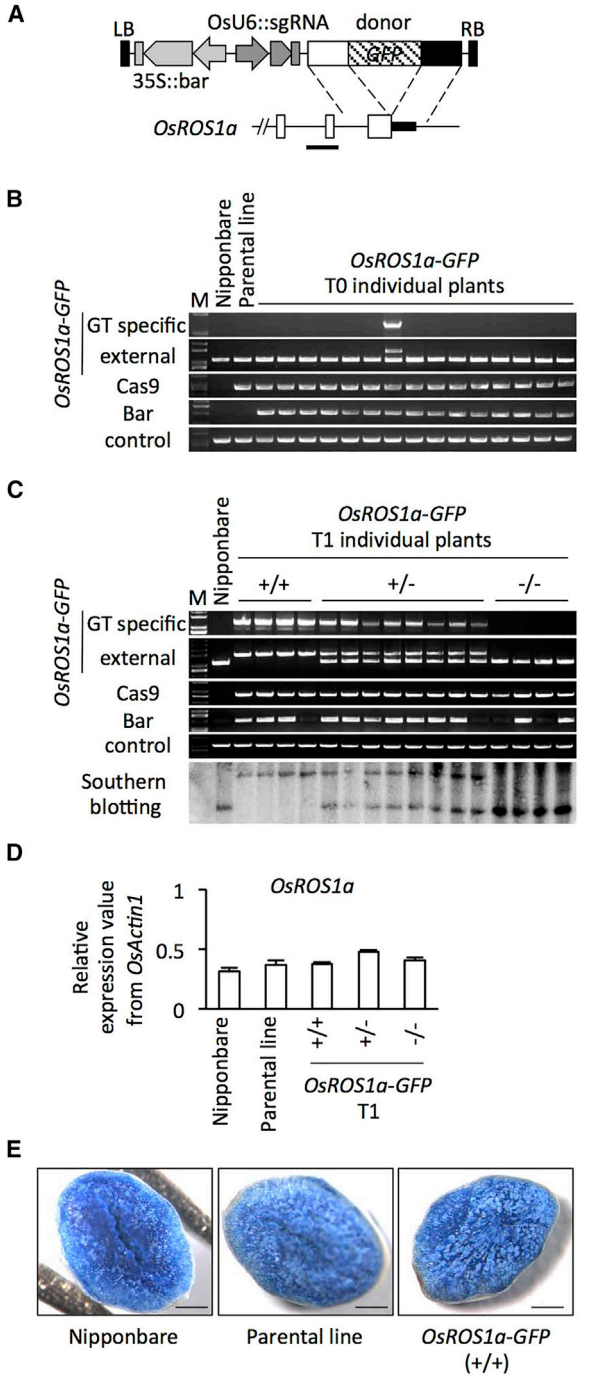

Figure 3. *GFP* knockin into the *OsROS1a* locus

(A) Schematic of the *OsROS1a-GFP* donor construct and endogenous target locus. The donor construct contains an in-frame *GFP* sequence with endogenous *OsROS1a* harboring 1,000 bp homology arms (Figure S1B). Horizontal line represents the probe for Southern blotting (Figure S1B).

(B and C) PCR genotyping and Southern blot hybridization of *OsROS1a-GFP* T0 (B) and T1 (C).

(D) qRT-PCR for *OsROS1a* expression in the T1 *OsROS1a-GFP* plants. The error bars indicate standard deviations (n = 4).

(E) Evans blue staining of transversally sectioned mature grains. The scale bar indicates 0.5 mm.

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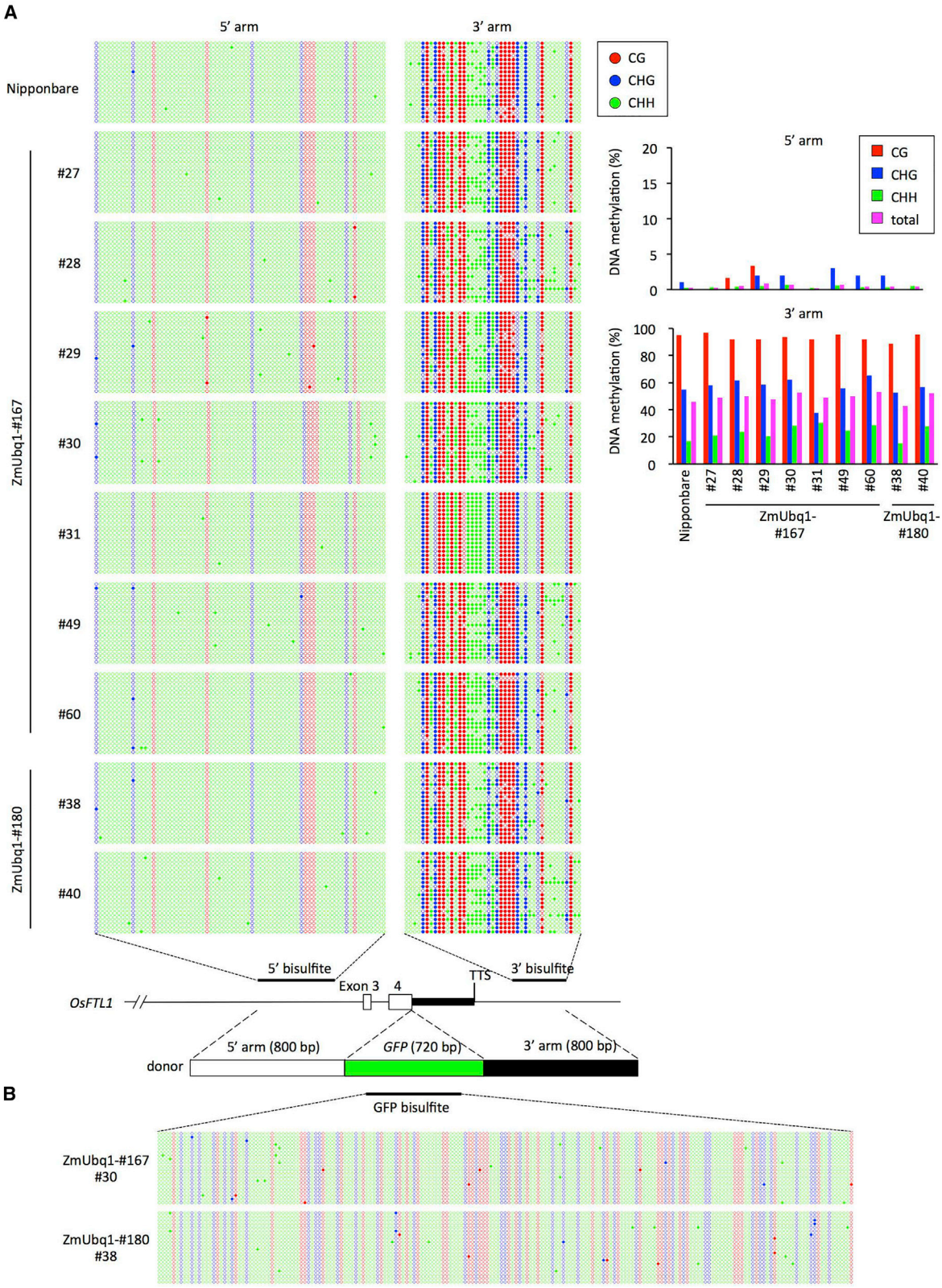

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in *Arabidopsis*. However, no alteration in DNA methylation at the *PPOX* locus in the other two lines, as well as other GT lines at the *CRU3* locus, was also observed in the same report.[40] Furthermore, DNA methylation was not altered, and *de novo* DNA methylation was not established at the GT loci in *Arabidopsis*.[23,27,35] These reports suggest that DNA methylation status is generally unaffected by GT events in *Arabidopsis*. In this study, we examined whether DNA methylation at homology arm regions was altered by CRISPR-Cas9-mediated GT in rice. We speculated that DNA methylation could be changed at the GT targeting region due to callus-mediated *Agrobacterium* transformation and its tissue culture process. Rice plants regenerated from calli display altered DNA methylation profiles.[44] However, our individual bisulfite sequencing results of the 5′ and 3′ homology arm regions in T0 *OsFTL1-GFP* knockin plants clearly indicate that GT events did not affect DNA methylation status in rice.

In the current study, we examined *GFP* knockin into two different endogenous loci, *OsFTL1* and *OsROS1a,* in rice. Our sequential transformation method does not rely on any selection markers at the GT target site. In *Arabidopsis*, we have recently shown that all transgenes, including Cas9 from the parental line and the donor from the second transformation, can be removed by backcrossing.[35] Indeed, donor transgene-free *OsFTL1-GFP* and *OsROS1a-GFP* GT rice plants in the T1 generation were obtained in this study. This result strongly indicates that all transgenes, which are required for GT establishment, can be readily removed by backcrossing. Recently, GABA-enriched tomato, which was generated via CRISPR-Cas9-mediated mutagenesis, but does not contain the Cas9 transgene in the final products, has been released to consumers.[45] GT technology can modify gene function and expression by either sequence replacement or knockin. When genomic sequences from a plant that can interbreed with the host crop are used as the donor for GT, the resulting transgene-free GT crop may be considered as nontransgenic, and thus could enter the consumer market rapidly.

Limitation of the study

This study demonstrated that it is possible to perform GT in rice using the sequential transformation strategy, which was more efficient than the all-in-one method. However, sequential transformation requires parental lines with high Cas9 activity, which is time-consuming and limits its applicability to diverse genetic backgrounds. Continued improvements in GT technology in plants will further facilitate the establishment of trait-improved crop plants through molecular breeding.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2022.100389.

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AUTHOR CONTRIBUTIONS

W.X.Z., R.W., D.K., F.P., and D.M. designed the research; W.X.Z., R.W., D.K., and F.P. performed the experiments with support from M.C., W.J.Z., F.G., H.Z., and Z.W.; M.C. and S.H. generated transformants; D.M. supervised the project; W.X.Z., R.W., D.K., J.K, J.K.Z., F.F., and D.M. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Figure 4. Epigenetic effects at the GT target *OsFTL1* locus

Individual locus bisulfite sequencing analysis.

(A) Bisulfite sequencing at the 5′ and 3′ homology arms is shown for *OsFTL1-GFP* T0 rice plants. Purple, total C methylation; red, CG methylation; blue, CHG methylation; green, CHH methylation.

(B) Individual bisulfite sequencing at the knockin *GFP* sequence. At least 20 independent clones were sequenced for each sample for bisulfite sequencing analysis. Sequencing results were analyzed using Kismeth.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
cetyltrimethyl ammonium bromide	Sigma	Cat# 57-09-0
SpeI	New England BioLabs	Cat# R3133S
Critical commercial assays		
DNA labeling kit	Takara	Cat# 6045
RNeasy Plant mini kit	Qiagen	Cat# 74904
Trans-Script II	Trans-Gen Biotech	Cat# AH301-02
ChamQ SYBR qPCR Master Mix	Vazyme	Cat# Q311-02
Experimental models: Organisms/strains		
Rice parental line: #167 ZmUbq1 pro::SpCas9	This paper	N/A
Rice parental line: #180 ZmUbq1 pro::SpCas9	This paper	N/A
Rice, OsFTL1-GFP KI lines: #167-27, 28, 29, 30, 31, 49, 60 #180-38, 40	This paper	N/A
Rice, OsROS1a-GFP KI line	This paper	N/A
Oligonucleotides		
See Table S1 for all oligonucleotides used in this study	N/A	N/A
Recombinant DNA		
pCambia1300	Addgene	Cat# 5930
pCambia3301	Biofeng	Cat# I47-825O-882O
ZmUbq1 pro::SpCas9 parental line construct (pCambia1300 background)	This paper	N/A
OsFTL1-GFP KI construct (pCambia3301 background)	This paper	N/A
OsROS1a-GFP KI construct (pCambia3301 background)	This paper	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daisuke Miki (daisukemiki@psc.ac.cn).

Materials availability

- This study did not generate new unique reagents.
- All plasmid and rice lines generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- All data will be shared by the lead contact upon request after publication.
- No original code was generated in this study.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Rice

The Japonica rice *Oryza sativa* cultivar Nipponbare was used in the study. Rice plants were grown at 28°C in soil with a 12 h light/12 h dark photoperiod in a greenhouse.

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METHOD DETAILS

Plant materials and transformation

The Japonica rice *O. sativa* cultivar Nipponbare was used for all experiments. All plants were grown at 28°C in soil with a 12 h light/12 h dark photoperiod in a greenhouse. *Agrobacterium*-mediated transformation of rice was performed as reported previously.[46]

Plasmid construction

The optimized coding sequence of the *S. pyogenes* Cas9 (hSpCas9) (CRISPR/Cas9) plasmid was previously reported,[34] and the parental line construct was generated with the maize *Ubiquitin 1* (ZmUbq1) promoter[32] in a binary vector, pCambia1300. The OsU6 promoter was used to drive sgRNA as previously reported.[2] The donor constructs for GT were made following the reported procedure in the pCambia3301 background.[36]

DNA analysis

Total DNA was extracted by the cetyltrimethyl ammonium bromide (CTAB) method from T0 mature leaves or 2-week-old T1 seedlings. Extracted DNA was used for analysis of mutant genotyping by restriction length polymorphism (RFLP) and qChop-PCR. For RFLP analysis in the potential parental lines, the genetic region surrounding the sgRNA target site was amplified by PCR followed by SpeI restriction enzyme digestion. The polymorphism was detected by agarose gel electrophoresis. The PCR products without restriction enzyme digestion were sequenced to determine mutations. Extracted DNA was digested with SpeI restriction enzyme followed by qPCR for qChop-PCR analysis.

Southern blotting was performed according to published protocols (Miki et al., 2018). Briefly, extracted 2 μg DNA was digested overnight with chosen restriction enzymes (Figure S1), then separated on a 1.2% agarose gel, visualized by Image Lab Software and Gel Doc XR (BIO-RAD), and then transferred to nylon membrane (GE healthcare). The probes (Figure S1) were labeled with 32P-α-dCTP by using the Random primer DNA labeling kit (Takara). The hybridization signals were detected with a phosphor imager (Fuji).

RNA analysis

Total RNA was extracted from callus tissues by using an RNeasy Plant mini kit (Qiagen) and reverse transcribed by Trans-Script II (Trans-Gen Biotech) with oligo (dT) primers. One microliter of RT product was used as a template for qRT–PCR.

QUANTIFICATION AND STATISTICAL ANALYSIS

The mutation ratio in the parental lines was determined by qChop-PCR (Figure 1C, gray bar). The relative Cas9 expression value was calculated from *OsAct1* (Figure 1C, black bar). The error bars indicate the SD for n = 3 experimental replicates. The relative *OsROS1a* expression from *OsAct1* was determined in the T1 *OsROS1a-GFP* plants by qRT–PCR (Figure 3D). The error bars indicate the SD for n = 4 experimental replicates.