



Dual-targeting by CRISPR/Cas9 leads to efficient point mutagenesis but only rare targeted deletions in the rice genome

Bhuvan Pathak^{1,2} · Shan Zhao¹ · Muthusamy Manoharan³ · Vibha Srivastava^{1,2,4}

Received: 5 October 2018 / Accepted: 20 March 2019 / Published online: 28 March 2019
© The Author(s) 2019

Abstract

The present study investigated the efficiency of CRISPR/Cas9 in creating genomic deletions as the basis of its application in removing selection marker genes or the intergenic regions. Three loci, representing a transgene and two rice genes, were targeted at two sites each, in separate experiments, and the deletion of the defined fragments was investigated by PCR and sequencing. Genomic deletions were found at a low rate among the transformed callus lines that could be isolated, cultured, and regenerated into plants harboring the deletion. However, randomly regenerated plants showed mixed genomic effects, and generally did not harbor heritable genomic deletions. To determine whether point mutations occurred at each targeted site, a total of 114 plants consisting of primary transgenic lines and their progeny were analyzed. Ninety-three plants showed targeting, 60 of which were targeted at both sites. The presence of point mutations at both sites was correlated with the guide RNA efficiency. In summary, genomic deletions through dual-targeting by the paired-guide RNAs were generally observed in callus, while de novo point mutations at one or both sites occurred at high rates in transgenic plants and their progeny, generating a variety of insertion–deletions or single-nucleotide variations. In this study, point mutations were exceedingly favored over genomic deletions; therefore, for the recovery of plant lines harboring targeted deletions, identifying early transformed clones harboring the deletions, and isolating them for plant regeneration is recommended.

Keywords CRISPR/Cas9 · Multiplex gene editing · Genomic deletion · Point mutations · Genome engineering

Introduction

Genome-editing effects are based on the creation of double-stranded breaks (DSB) in the target DNA that are repaired by the cell through non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways (Jasin and Haber 2016; Waterworth et al. 2011). While HDR leads to predictable outcomes as determined by the DNA template, NHEJ ends up with insertions, deletions and/or substitutions (Puchta et al. 1996; Rouet et al. 1994; Szostak et al. 1983),

leading to gene knockouts. The power of CRISPR/Cas9 lies in its efficiency in creating DSBs in genomic sequences containing NGG protospacer adjacent motif (PAM). The simplified version of CRISPR/Cas9 consists of a single-guide (sg) RNA bound to Cas9 (sgRNA:Cas9) that targets genomic sequences through RNA–DNA pairing. Although, sgRNA design is based on a relatively simple 5'-N(20)-NGG-3' targeting rule (Cong et al. 2013; Jinek et al. 2012; Mali et al. 2013; Mojica et al. 2009), the efficiency of different sgRNAs could vary in the cell. Therefore, multiple sgRNAs are often used in creating targeted knockouts. As a result, targeted genomic deletions by CRISPR/Cas9 have been observed in numerous studies.

Dual-targeting by CRISPR/Cas9, based on the paired use of sgRNAs, could generate somatic and heritable deletions of genomic fragments. Short deletions of ~100 bp are frequently reported in plants (Brooks et al. 2014; Kapusi et al. 2017; Nekrasov et al. 2017; Ordon et al. 2017). Dual-targeting was also effective in deleting larger fragments (~0.5 kb, ~0.7 kb, and 1.6 kb) as reported in maize, kiwi fruit, and rice (Minkenberg et al. 2017; Shi et al. 2017;

✉ Vibha Srivastava
vibhas@uark.edu

¹ Department of Crop, Soil and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701, USA

² Cell and Molecular Biology Program, University of Arkansas, Fayetteville, AR 72701, USA

³ Department of Agriculture, University of Arkansas at Pine Bluff, Pine Bluff, AR 71601, USA

⁴ Department of Horticulture, University of Arkansas, Fayetteville, AR 72701, USA

Srivastava et al. 2017; Wang et al. 2018). Fragments of 10–12 kb could be deleted in rice and *Arabidopsis* (Durr et al. 2018; Wang et al. 2017a), and even larger fragments of 170–245 kb were deleted by multiplex targeting in rice (Zhou et al. 2014). The efficiencies of genomic deletions varied greatly in these reports, but short deletions (~ 100 bp) were obtained more readily than large deletions (Kapusi et al. 2017; Ordon et al. 2017; Wang et al. 2017a). However, compared to point mutagenesis (effect of a single sgRNA), genomic deletions (effect of paired sgRNAs) consistently occurred at much lower rate even when two or more sgRNAs of equal efficiencies were used (Minkenberg et al. 2017; Tian et al. 2017; Wang et al. 2017a, b).

The application of CRISPR/Cas9 in genome editing is limited by the DNA repair pathways of the host organism. In somatic cells of plants and other higher organisms, NHEJ is the major repair pathway (Puchta et al. 1996; Waterworth et al. 2011); therefore, targeted mutagenesis is the most successful application of CRISPR/Cas9. Another genomic effect that could be created by NHEJ is fragment deletion by a pair of sgRNAs to simultaneously create DSBs at two different sites on a segment of the genome (dual-simultaneous targeting). Ligations of the two distal ends through NHEJ would effectively delete the intervening fragment. Genomic deletions could serve as useful editing effects in functional genomics and biotechnology by targeting gene clusters, *cis*-regulatory elements or transgenes. However, current understanding of dual-targeting by CRISPR/Cas9 in creating genomic deletions is narrow. Many studies have reported genomic deletions, but little is known about the efficiency and success in recovering stable plants lines harboring the defined deletion.

The present study investigated the efficiency of obtaining defined genomic deletions of 240 bp, 945 bp, and 1637 bp from three different loci by dual-targeting in rice. Defined deletions were detected by PCR among transformed calli, and as expected, plants regenerated from these calli harbored the deletions and transmitted to their progeny. However, randomly regenerated plants harboring mixed genomic effects either did not show deletions or showed a low rate of somatic deletions. Furthermore, while targeting frequency of each sgRNA increased in the progeny, genomic deletions remained undetectable. Therefore, for ensuring the recovery of plant lines harboring deletions defined by dual-targeting, it is recommended to screen early transgenic clones (calli) and isolate the characterized clones for plant regeneration. The recovery of *de novo* deletion lines through plant screening and progeny analysis, at least in rice, appears to be highly unlikely.

Results

Experimental design

The efficiency of CRISPR/Cas9 in deleting genomic fragments was estimated on three loci, *GUS* transgene (AF485783), rice *PDS* (LOC_Os03g08570), and rice *Chalk5* (LOC_Os05g06480.1; Chromosome 5: 3,335,405–3,341,600) (Fig. 1a). Two sites in each locus were chosen based on 5'-N(20)-NGG-3' rule (Cong et al. 2013; Jinek et al. 2012; Mali et al. 2013), with the goal of creating deletions through simultaneous targeting by a pair of sgRNAs (sg1 + sg2). While *GUS* and *PDS* sgRNAs targeted the genic regions, *Chalk5* sgRNAs targeted an intergenic region harboring *cis*-regulatory elements (Fig. 1a). To generate sg1 and sg2 from a single vector, oligonucleotides containing sgRNA spacers were cloned in pRGE32, which contains tRNA splicing mechanism to generate multiple sgRNAs from a single transcript produced by the rice *U3* promoter (Xie et al. 2015). The resulting *GUS*-, *PDS*- or *Chalk5*- targeting vectors, pJU24, pJU34, and pJU46, respectively, were transformed into the B1 rice line, expressing the *GUS* gene, or the wild-type Nipponbare rice. Line B1 that contains a single-copy of *GUS* gene has been described earlier (Nandy and Srivastava 2012). The resulting transgenic lines were screened by PCR to identify deletions in *GUS*, *PDS*, or *Chalk5* genes, indicated by amplification of fragments shorter by 1637 bp, 987 bp, and 240 bp, respectively (Fig. 1a). A representative PCR indicating genomic deletion in the three loci is shown in Fig. 1b. Targeted deletion of *GUS* in the callus lines has been described earlier (Srivastava et al. 2017). This work further investigated genomic deletions on two more loci, *PDS* and *Chalk5*, and analyzed plant lines to determine the rates of genomic deletions and point mutations through amplicon sequencing by the Sanger method.

Detection of genomic deletions in callus lines

Genomic deletions (Δ) in the callus lines transformed with pJU24, pJU34, or pJU46 were tested by PCR and indicated by the respective Δ amplicons observed in a PCR (Fig. 1b). As reported earlier, *GUS* deletion in pJU24-transformed lines occurred in 2 out of 113 callus lines (Srivastava et al. 2017). In the present study, genomic deletions in two additional loci, *PDS* and *Chalk5* loci, were determined in pJU34- and pJU46-transformed lines (Table 1). Genomic deletions at *PDS* locus was found in 2 out of 32 callus lines and at *Chalk5* locus in 4 out of 53 callus lines. Sequencing of the Δ amplicons indicated that the distal ends, created by the blunt DSBs, ligated

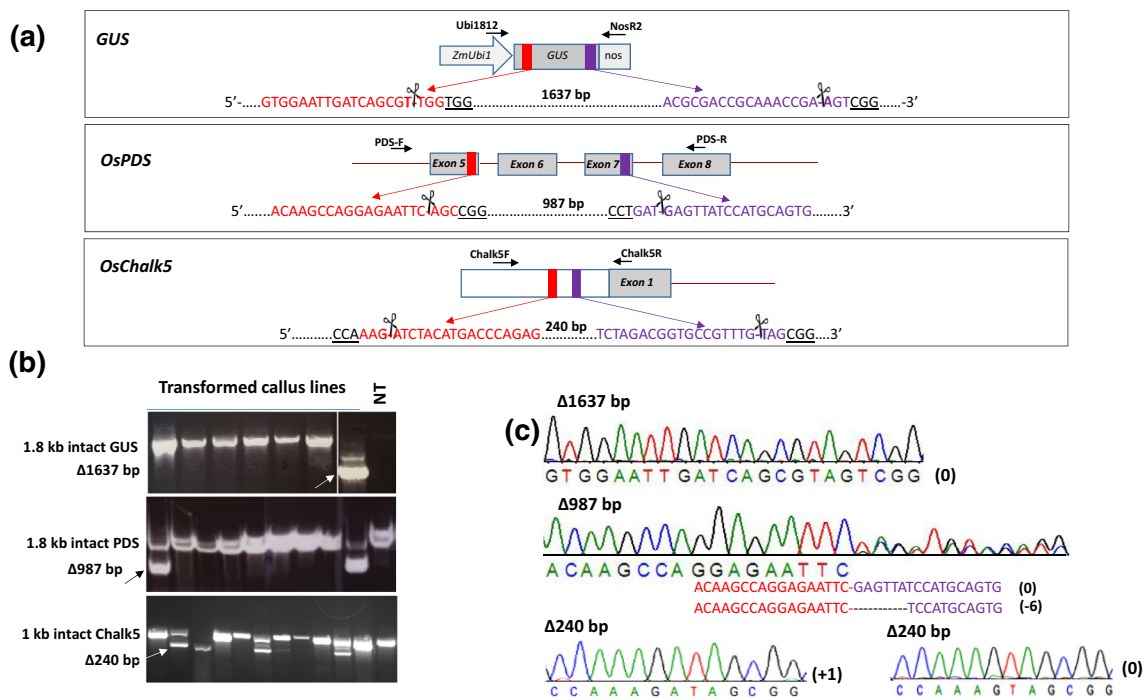


Fig. 1 Dual-targeting by CRISPR/Cas9 for fragment deletions. **a** Paired sgRNAs for targeting three genes, transgene *GUS* and native genes, *OsPDS* and *OsChalk5*, in rice. Full structure of *GUS* gene and partial structures of *OsPDS* and *OsChalk5* genes are shown with sgRNA (red and purple boxes) and primer (arrows) locations. sgRNA spacer 1 (red) or sgRNA spacer 2 (purple) for each locus are shown with protospacer adjacent motif (PAM) (underlined). The positions of double-strand break (DSB) sites are shown by scissors that defined deletion sizes given in base pairs (bp). *ZmUbi* refers to maize Ubiquitin-1 promoter and *nos* to nopaline synthase 3' transcription

terminator. *GUS* and *OsPDS* genes are targeted in the genic regions (exons), while *OsChalk5* in the intergenic region, upstream of promoter harboring *cis*-elements (white box). **b** PCR screening of callus clones using forward and reverse primers spanning targeted sites (see Table 1; **a**). Representative callus lines are shown with non-transgenic controls (NT; cv. Nipponbare). The intact and the deletion fragments (Δ) are indicated; **c** Sequences of the representative deletion fragments of *GUS* ($\Delta 1637$ bp), *PDS* ($\Delta 987$ bp), and *Chalk5* ($\Delta 240$ bp) loci. The number of bases representing insertion-deletions (indels) is given in parentheses

Table 1 Genomic deletion by dual-targeting in callus lines

Exp.	Target Gene	Vector	Predicted Δ size (bp)	Total lines	PCR detection	DNA sequencing		Eff. (%) ^a
						(-) InDel	(+) InDel	
1	<i>OsPDS</i>	pJU34	985	32	2	–	2	6.2
2	<i>OsChalk5</i>	pJU46	240	53	4	2	2	7.5

^aPercent events showing genomic deletion by PCR as shown in Fig. 1. *GUS* deletion data is given in Srivastava et al. 2017

without indels or with short indels to generate the Δ locus. The indels generally consisted of insertion or deletion of a single nucleotide or a few nucleotides (Fig. 1c), which is consistent with other studies that report single-nucleotide variations as most common outcome of CRISPR/Cas9 targeting (Mao et al. 2013, van Overbeek et al. 2016). One of the pJU46 lines (*Chalk5*) showed an amplicon ~0.2 kb larger than the intact *Chalk5* amplicon. Sequencing of this amplicon showed insertion of 0.2 kb fragment of unknown source in one of the targeted sites (single-site targeting, data not shown). Overall, the efficiency of creating genomic deletions by dual-targeting was low and variable

with the sgRNA pairs (sg1 + sg2). Targeted deletions by *GUS* sgRNA pairs were reported in only 1.7% of the transformed callus lines (Srivastava et al. 2017). The *PDS* and *Chalk5* sgRNA pairs, on the other hand, generated significantly higher rates of deletion at somewhat similar rates in the callus lines (Table 1). Nevertheless, these observations indicate that genomic deletions could be created through dual-targeting by CRISPR/Cas9, and as reported earlier, calli harboring Δ locus could be regenerated into plants (Srivastava et al. 2017). Plants regenerated from one of the callus lines (line#72) contained homozygous Δ locus, indicated by the presence of $\Delta 1637$ bp amplicon and absence

of 1.8 kb amplicon in the PCR. As expected, the progeny of this plant inherited the stable Δ locus that independently segregated from *Cas9* (Fig. 2a). The sequence of the Δ 1637 bp in these plants was consistent with the creation of DSB at the predicted sites (3-bp upstream of PAM in each targeted site) followed by ligation of the distal ends without indels (Fig. 2b).

Targeting efficiency in plants

As described above, plant lines carrying the defined Δ locus could be regenerated from calli harboring the deletion. In the same experiment, a number of chimeric T0 plants were also regenerated that showed somatic deletions indicated by the presence of two amplicons, indicative of intact locus and Δ locus, in the same PCR reaction (Srivastava et al. 2017). However, when these chimeric plants were analyzed at a later stage of growth (flowering) in the

greenhouse, the Δ 1637 bp amplicon was undetectable, in spite of testing multiple tissue from different tillers of each plant. This observation suggests that the young regenerated plants harbored somatic deletions that are unlikely to be transmitted to the progeny. Among *PDS* and *Chalk5* T0 plants, genomic deletions were undetectable by PCR at both early and late stages of growth (data not shown). To investigate the individual effect of each sgRNA, T0 plants were characterized for the presence of point mutations at each targeted site. A total of 50 T0 plants, representing *GUS*, *PDS*, or *Chalk5* targeting were analyzed by PCR and sequencing (Table 2). Some of these *GUS* plants selected for this analysis showed Δ 1637 bp amplicon in the leaf tissue of the young regenerated plants (Srivastava et al. 2017). Twelve of the 21 *GUS* plants did not show mutations at either targeted sites. The remaining nine showed targeting but only at sg2 target. Of the 12 *PDS* lines, 3 lacked targeting, while 9 contained targeting at both sites. Finally, 6 out of 17 *Chalk5* lines lacked targeting, and the remaining contained targeting at both sites (Table 2). T0 plants were mostly chimeric for targeting, as 2 or more traces were observed in the characteristic superimposed overlapping peaks downstream of the DSB site in the sequencing spectra. Analysis of these traces revealed the types of mutations found at the DSB sites (Fig. 3). In summary, targeting efficiency of the two *GUS* sgRNAs was highly dissimilar, but the two *PDS* or *Chalk5* sgRNAs showed similar targeting efficiency (Table 2). Sequence alignments of the targeted sites revealed interesting observations: (1) the targeted *GUS* site in all 9 T0 plants contained only a single-nucleotide variation consisting of 1 bp insertion, deletion or substitution at the predicted DSB site; (2) the two targeted *PDS* sites contained short deletions ranging from 1 to 7 bp, with only one line containing a larger deletion; and (3) the targeted *Chalk5* sites showed most diverse types of mutations with short indels and 1 bp insertions at the two DSB sites (Fig. 3). These observations suggest that possibly genomic context, target sequence, and sgRNA efficiency influence the outcome of CRISPR/Cas9 targeting. In support, a recent study in yeast showed that types of indels generated by CRISPR/Cas9 depended on DNA sequence context and PAM orientation (Lemos et al. 2018).

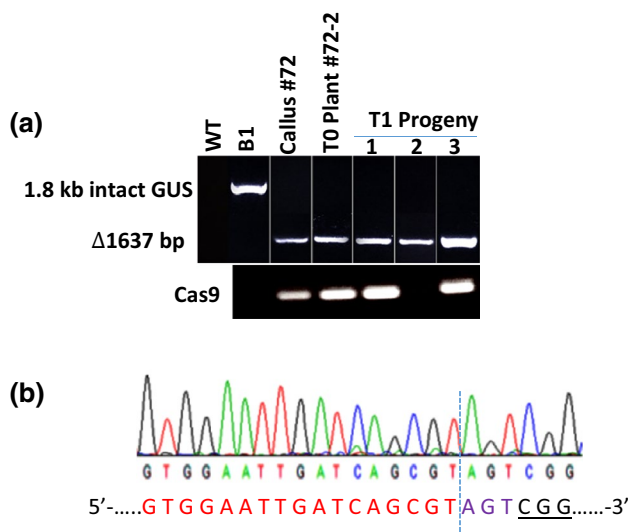


Fig. 2 Recovery of stable plant lines harboring Δ 1637 bp *GUS* deletion. **a** PCR analysis to detect *GUS* and *Cas9* in the callus, primary transgenic plant (T0), and the progeny (T1). WT, wild-type Nipponbare; B1, transgenic *GUS* line; **b** DNA sequencing spectrum of Δ 1637 bp fragment in T0 plant#72-2 generated by the paired used of sgRNAs. The observed sequence matches the predicted deletion site derived from joining of distal ends without indels. Dashed vertical line indicates blunt DSB ligation

Table 2 Point mutations in primary transgenic (T0) plants

Exp.	Target	Total no. of plants	Non-targeted	No. of plants targeted ^a		Eff. (%) ^b	
				sg1 site	sg2 site	sg1	sg2
1	<i>GUS</i>	21	12	0	9	–	42
2	<i>OsPDS</i>	12	3	9	9	75	75
3	<i>OsChalk5</i>	17	6	11	11	64	64

^aGenerally chimeric mutations observed. Types of mutations shown in Fig. 3

^bPercent plants harboring mostly chimeric mutations at predicted DSB sites

	sg1 target	sg2 target	Ref
GUS	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA-AGTCCG	(0) (-1)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCG--AGTCCG	(0) (+1)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGAaAGTCCG	(0) (A>G)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGG-AGTCCG	(0) (+1)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGAaAGTCCG	(0) (+1)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCG--AGTCCG	(0) (-1)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGG-AGTCCG	(0) (A>G)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA-AGTCCG	(0) (G>C)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGAaAGTCCG	(0) (+1)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGAaAGTCCG	(0) (+1)
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGAcAGTCCG	(0) (+1)
PDS	CCAAACAAGCCAGGAGAATTC-AGCCGGTTTG.....	CCTGAT-GAGTTATCCATGCAGTGCATTCT	Ref
	CCAAACAAGCCAGGAGAA-----CGGTTTG.....	CCTGAT-----CCATGCAGTGCATTCT	(-6) (-7)
	CCAAACAAGCCAGGAGAATTC-----CGGTTGG.....	CCTGAT-----GTTATCCATGCAGTGCATTCT	(-3) (-2)
	CCAAACAAGCCAGGAGAATTC-----CGGTTTG.....	CCTGAT--AGTTATCCATGCAGTGCATTCT	(-2) (-1)
Chalk5	CCA AAG-ATCTACATGACCCAGAGTGTTTTATC.....	AAGGATTCTAGACGGTGCCGTTTG-TAGCCG	Ref
	CCA AAG-accataaccgatataTG-----C.....	AAGGATTCTA-----GCGG	(±24) (-16)
	CCA AAG-cacagggttctgt-----GTTTATC.....	AAGGATTCTggcggaacggaTTG-TAGCCG	(±18) (+12)
	CCA AA--ccataac-----TGTTTATC.....	AAGGATTCTAGAC-----GCGG	(±18) (-13)
	CCA -----aaaccataaccgat-ATC.....	AAGGATTCTAGACGGTGCCGTTTGaTAGCCG	(±26) (+1)
	CCA AAG-----TACATGACCCAGAGTGTTTTATC.....	AAGGATTCTAGACGGTGCCGTTTGaTAGCCG	(-3) (+1)
	CCA AAG-ta-----GACCCAGAGTGTTTTATC.....	AAGGATTCTAGACGGTGCCGTTTgTAGCCG	(±8) (+1)

Fig. 3 Types of mutations observed in T0 plants. Sequence alignments of *GUS*, *PDS* and *Chalk5* sequences at sg1 and sg2 targeted sites (yellow highlights). PAM sequences are underlined, and DSB site is shown as (-) in each reference sequence. Insertion/deletions/

substitutions for each site are shown on the right. Deletions are shown as red dashes, insertions as small red letters, and substitutions as large blue letters

Targeting in progeny plants

To investigate inheritance of CRISPR-induced deletions, 61 progeny seedlings derived from three *GUS* T0 plants were analyzed by PCR. None of the progeny, however, showed $\Delta 1637$ bp amplicon, indicative of stable genomic deletion. These plants were also stained for *GUS* activity, 34 of which were negative, indicating targeting at sg1 and/or sg2 sites. To determine the inheritance of point mutations, selected *GUS*-negative progeny derived from a single parent plant was analyzed and compared with the parent plant that contained chimeric targeting at sg2 site. In the parent plant, no targeting was evident in sg1 site, but three types of mutations were observed at the predicted sg2 DSB site: +1 (A or C) and A-to-C substitution (Fig. 4a); however, +1 C was the most commonly observed mutation in multi-sample analysis that likely rendered the plant *GUS* negative. None of the T1 plants showed $\Delta 1637$ bp amplicon; however, de novo targeting by sg1 was frequently observed. Eight of the 17 T1 plants showed chimeric targeting (≥ 2 types of sequences) at sg1 target. The most common type of mutation at sg1 target was 1 bp deletion; however, 1 bp insertion and longer deletions were also observed (Fig. 4a). The analysis of sg2 target among T1 plants revealed that all 17 plants contained mono-allelic or biallelic mutations (Table 3). Biallelic mutations were either identical on each allele (homozygous) or different (heterozygous). The alignment of sequences revealed

that all observed mutations were also present in the parent. Four T1 plants (T1-7, 9, 12, 15) had segregated from Cas9 gene, confirming inheritance of the mutation (Fig. 4a). In summary, while targeting at both sites was observed in T1 plants, de novo genomic deletions were undetectable.

Next, T2 progeny derived from three T1 plants (T1-2, T1-3, and T1-4) were analyzed by PCR and sequencing. Once again, no genomic deletion was detected in any of the T2 plants. The three T1 parents all contained identical mutation at sg2 site (+1 C), but differed at sg1 site. T1-2 contained 7 bp deletion at sg1 site, but its progeny completely lacked mutations at sg1 sites and contained de novo single-nucleotide variation (+1 A) at sg2 site, indicating that mutations observed in the parent were not heritable and de novo mutations were introduced. T1-3 lacked mutations at sg1 site and contained C insertion at sg2 site. Its T2 progeny showed de novo mutations at sg1 site: single bp variation (insertion/deletion/substitution) and 6 bp deletion, whereas at sg2 site, both inheritances of +1 C insertion and de novo single-base variations were observed. T1-4 contained -1 T in sg1 site and +1 C at sg2 target. Its T2 progeny, one of which lacked Cas9, inherited these mutations; however, new mutations were also observed: +1 A and A-C substitution (Fig. 4b). All of these mutations were observed in the T1 parents; therefore, mutations at sg2 target were likely inherited, but de novo mutations were also created. Inheritance of mutation was confirmed in one T2 plant that contained -1

(a)

	sg1 target	sg2 target	sg1	sg2	Cas9
<u>T0</u>	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	
<u>Parent</u>	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA a AGT C GG	(0)	(+1)	
	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA- C GT C GG	(0)	(A>C)	
T1-1	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA a AGT C GG	(0)	(+1)	+
T1-2	GTGGAATTGATCAGCG- --- ---GAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(-7)	(+1)	+
T1-3	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	+
T1-4	GTGGAATTGATCAGCGT- GG TGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(-1)	(+1)	+
T1-5c	GTGGAATTGATCAGCGT- --- TGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(-3)	(+1)	+
T1-6	GTGGAATTGATCAGCGT- CGG TGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(T>C)	(+1)	+
T1-7	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	-
T1-8	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	+
T1-9	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	-
T1-10	GTGGAATTGATCAGCGT a TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(+1)	(+1)	+
T1-11	GTGGAATTGATCAGCG- --- TGGTGGGAAA.....	TCGCGACCGCAAACCGA a AGT C GG	(-1)	(+1)	+
T1-12	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	-
T1-13	GTGGAATTGATCAGCG- --- TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(-1)	(+1)	+
T1-14	GTGGAATTGATCAGCG- --- TGGTGGGAAA.....	TCGCGACCGCAAACCGA- C GT C GG	(-1)	(A>C)	+
T1-15	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA a AGT C GG	(0)	(+1)	-
T1-16	GTGGAATTGATCAGCGT a TGGTGGGAAA.....	TCGCGACCGCAAACCGA c AGT C GG	(+1)	(+1)	+
T1-17	GTGGAATTGATCAGCGT-TGGTGGGAAA.....	TCGCGACCGCAAACCGA- C GT C GG	(0)	(A>C)	+

(b)

	sg1	sg2	sg1	sg2	Cas9
<u>T1-2</u>	GTGGAATTGATCAGCG- --- ---G.....	TCGCGACCGCAAACCGA c AGT C GG	(-7)	(+1)	+
<u>T2-1</u>	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA a AGT C GG	(0)	(+1)	+
<u>T1-3</u>	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	+
T2-2	GTGGAATTGATCAGCGT- AGG TGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(T>A)	(+1)	+
T2-3	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	+
T2-4	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA a AGT C GG	(0)	(+1)	+
T2-5	GTGGAATTGATCAGCGT- GG TGGG.....	TCGCGACCGCAAACCGA- C GT C GG	(-1)	(A>C)	+
T2-6	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA a AGT C GG	(0)	(+1)	+
T2-7	GTGGAATTGATCAGCGT t TGGTGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(+1)	(+1)	+
T2-8	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA- C GT C GG	(0)	(A>C)	+
T2-9	GTGGAATTGAT- --- ---TGGTGGG.....	TCGCGACCGCAAACCGA- C GT C GG	(-6)	(A>C)	+
T2-10	GTGGAATTGATCAGCGT- GG TGGG.....	TCGCGACCGCAAACCGA- C GT C GG	(-1)	(A>C)	+
T2-11	GTGGAATTGATCAGCGT- GG TGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(-1)	(+1)	+
T2-12	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	+
T2-13	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(0)	(+1)	+
<u>T1-4</u>	GTGGAATTGATCAGCGT- GG TGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(-1)	(+1)	+
T2-14	GTGGAATTGATCAGCGT- GG TGGG.....	TCGCGACCGCAAACCGA a AGT C GG	(-1)	(+1)	+
T2-15	GTGGAATTGATCAGCGT- GG TGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(-1)	(+1)	+
T2-16	GTGGAATTGATCAGCGT- GG TGGG.....	TCGCGACCGCAAACCGA c AGT C GG	(-1)	(+1)	-
T2-17	GTGGAATTGATCAGCGT-TGGTGGG.....	TCGCGACCGCAAACCGA- C GT C GG	(0)	(A>C)	+

Fig. 4 Genotyping of progeny plants derived from the T0 parent expressing GUS-targeting vector. **a** T1 progeny, and **b** T2 progeny. The mutation types in sg1 and sg2 targets are shown, see Fig. 3 for

notations. Bold T1/T2 lines are Cas9-negative. Parent plants are underlined with their representative progeny given below

Table 3 Point mutations in GUS-CRISPR/Cas9 progeny

Locus	Generation	No. of plants tested	sg1 mutations ^a				sg2 mutations ^a			
			Non-targeted	Mono-allelic	Bi-allelic ^b	Chimer. ^c	Non-targeted	Mono-allelic	Bi-allelic ^b	Chimer. ^c
GUS	T1	17	9	6	-	2	-	12	5	-
GUS	T2	17	8	7	1	1	-	10	7	-
Chalk5 ^d	T1	30	0	0	30	-	7	8	15	-

^aTypes of mutations shown in Figs. 4 and 5

^bHeterozygous or homozygous

^cPresence of > 2 overlapping traces downstream of DSB site in the sequencing spectra

^dT1 plants of Chalk5 from potentially same transgenic event but different T0 plants

and + 1 at the sg1 and sg2 sites, respectively (Fig. 4b). In summary, while genomic deletions remained undetectable, increased rate of point mutations (effect of single sgRNA) was observed in T1 and T2 progeny with single-base variation as the common type of mutation at the targeted site. We also investigated whether single-base variations frequently found at sg2 site could alone confer *GUS* negative phenotype as observed in T0 parent plant. We found that A–C substitution did not change the protein sequence, but + 1 A and + 1 C generated frame shift and early stop codon (data not shown), mutating the C-terminal catalytic domain of β -glucuronidase (*GUS*) enzyme (Wallace et al. 2010), leading to inactivation of *GUS* activity.

We also analyzed T1 progeny of *Chalk5* T0 plants that showed chimeric effects at sg1 and sg2 sites by superimposed overlapping peaks downstream of the DSB site in the sequencing spectra. The analysis of the spectra by CRISP-ID tool identified short deletions at sg1 site and 1 bp insertions (+ 1) at sg2 site (Fig. 5). Thirty T1 plants from this chimeric parent were analyzed by PCR and sequencing. No deletion was evident, but point mutations at each site were found as homozygous or heterozygous mutation (Table 3; Fig. 5). Furthermore, at least one of the mutations identified in the parent plant (– 3 at sg1 and + 1 at sg2) was transmitted to the progeny at high rate.

Same mutation pattern from different targeting events

We frequently observed – 1 and/or + 1 mutations at *GUS* sg1 and sg2 sites in the targeted lines. To investigate whether the same type of mutation arises from different targeting events, we compared *GUS* sg1 and sg2 sites in 23 different lines obtained from 3 different experiments. At sg1 site, deletion of a single nucleotide (– 1) at the DSB site was observed 13

times (Fig. 6a), whereas at sg2, insertion of a single nucleotide (+ 1) at the DSB site was observed 12 times (Fig. 6b). The next most frequent type of mutation was single-base substitution (s1), which either occurred at the DSB site or in the PAM (Fig. 6a, b). Other types of mutations at the two sites included short deletions or single-nucleotide variations, which were generally observed once in the population. In summary, the repair of sg1 and sg2 DSB sites led to a predictable mutation pattern of – 1 or + 1 in ~ 50% of the transformed lines generated within the experiment or between experiments.

Discussion

Plant genome engineering involves a variety of genomic modifications including gene insertion, replacement, inactivation, or deletion. Creating predictable genetic variation is highly desirable, but often defeated by the host repair processes that ignore DNA homologies and generate unpredictable mutations in higher plants (Jasin and Haber 2016; Puchta et al. 1996; Waterworth et al. 2011). As a result, targeted knockout is the most common outcome of genome editing. Genomic deletions, however, do not rely on homology-based DNA repair and, therefore, should be possible to create by standard gene-editing methods.

One of the applications of targeted genomic deletion is transgene excision to rid transgenic plant of antibiotic-resistance marker genes. While effective methods of transgene removal are available, they require specialized vector constructions, e.g., adding recombination sites or separating marker gene from the gene-of-interest in two T-DNAs (Gidoni et al. 2008; Komari et al. 1996; Wang et al. 2011). On the other hand, CRISPR/Cas9 can target loci by virtue of the cloned sgRNA spacers (Cong et al. 2013; Jinek et al.

	sg1 target	sg2 target	Ref	
Parent	CCA AAG-ATCTACATGACCCAGAG.....	TCTAGACGGTGCCGTTTG-TAG CCG		
	CCA AAG- ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTGaTAG CCG	(– 3)	(+ 1)
	CCA AAG- ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTG-TAG CCG	(– 3)	(0)
	CCA AAG-ta ---CATGACCCAGAG.....	TCTAGACGGTGCCGTTTG-TAG CCG	(± 5)	(0)
Progeny	CCA AAG- ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTGaTAG CCG	(– 3)	(+ 1)
	CCA AAG- ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTGgTAG CCG	(– 3)	(+ 1)
	CCA AAG- ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTG-TAG CCG	(– 3)	(0)
	CCA AAG^ta ---CtTGACCCACAG.....	TCTAGACGGTGCCGTTTG-TAG CCG	(± 6)	(0)
	CCtAgG^ ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTGaTAG CCG	(± 5)	(+ 1)
	CCA A- ^ ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTGaTAG CCG	(– 5)	(+ 1)
	CCA AAG^ ---TACATGACCCAGAG.....	TCTAGACGGTGCCGTTTGcTAG CCG	(– 3)	(+ 1)

Fig. 5 Genotyping of progeny plants derived from the T0 parent expressing *Chalk5*-targeting vector. The mutation types in sg1 and sg2 targets in the parent and progeny plants are aligned with the reference, see Fig. 3 for notations

Fig. 6 Frequency of mutations observed at *GUS* targets as determined by Sanger sequencing of the sg1 target (a) and sg2 target (b). The reference sequences with PAM (underlined) and DSB site (–) are shown on the top. Insertions (+) and deletions (–) are shown in red and substitutions (s) in blue fonts. s1 refers to single-nucleotide substitution at or near DSB site. Frequency refers to number of times a mutation type observed among the 23 lines. Boxed numbers indicate most common mutation types (–1 or +1) and their frequency

(a) Mutation types in sg1		ref	Freq.
GTGGAATTGATCAGCGT–TGGTGGG		ref	–
GTGGAATTGATCAGCG––TGGTGGG		–1	13
GTGGAATTGATCAGCGT a TGGTGGG		+1	2
GTGGAATTGATCAGCGT t TGGTGGG		+1	1
GTGGAATTGATCAGCGT–– G TGGG		–2	1
GTGGAATTGAT–––––TGGTGGG		–6	1
GTGGAATTGATCAGCG–––––G		–7	1
GTGGAATTGATCAGCGT––––TGGG		–3	1
GTGGAATTGATCAGCGT– CGG TGGG		s1	1
GTGGAATTGATCAGCGT– AGG TGGG		s1	1
GTGGAATTGATCAGCGT–TGG G GGG		s1	1
(b) Mutation types in sg2		ref	Freq.
TCGCGACCGCAAACCGA–AGT CGG		ref	–
TCGCGACCGCAAACCGA a AGT CGG		+1	6
TCGCGACCGCAAACCGA c AGT CGG		+1	3
TCGCGACCGCAAACCGA t AGT CGG		+1	3
TCGCGACCGCAAACCG––AGT CGG		–1	3
TCGCGACCGCAAACCG G –AGT CGG		s1	2
TCGCGACCGCAAACCGA–A CT CGG		s1	1
TCGCGACCGCAAACCGA– CGT CGG		s1	5

2012; Mali et al. 2013), thereby, giving more flexibility to the user. Genomic deletion could also be pursued to create null mutations to allow detection by standard PCR, while screening of small indels would require mismatch cleavage assay, DNA sequencing, quantitative, or digital PCR (Belhaj et al. 2013; Falabella et al. 2017; Kim et al. 2009; Voytas 2013; Xie and Yang 2013). Genomic deletions could also create useful traits. The natural variant of rice *DEP1* harbors $\Delta 625$ bp that confers erect panicles and increased grain yield (Huang et al. 2009), and the spontaneous deletions in maize *WAXY* gene alter starch composition of the grains (Wessler et al. 1990). Genomic deletions also play major roles in plant evolution (De Smet et al. 2017; Soltis et al. 2014). Divergence in the function of the duplicated genes could occur upon deletions in the genes (Haberer et al. 2004; Liu et al. 2011). For example, deletions in the intergenic regions could either remove or change the position of *cis*-elements leading to altered tissue specificity and neo-functionalization of the gene (Arsovski et al. 2015; De Smet and Van de Peer 2012). Thus, targeted genomic deletions could serve as useful effects in plant genome engineering.

CRISPR/Cas9 has emerged as the dominant gene-editing tool that holds a great promise for genome engineering in plants and animals. This study evaluated the practical application of CRISPR/Cas9 in creating targeted genomic deletions in three loci in the rice genome. Previously, we

reported successful deletion of *GUS* gene through dual-targeting by CRISPR/Cas9, which was accomplished by PCR screening and regeneration of the selected clones (Srivastava et al. 2017). Zhou et al. (2014) also reported chromosomal deletions in rice calli that were subjected to regeneration to recover plant lines. Similarly, in the present study, dual-targeting was successful in creating genomic deletion in transformed callus lines that mostly correlated with the efficiency of the sgRNA pairs. However, genomic deletions were rarely detected among plants transformed with Cas9:sgRNA constructs, and recovery of stable deletion lines was unsuccessful unless they were derived from calli harboring the deletion. This is somewhat surprising as point mutations by each sgRNA employed in dual-targeting occurred at high frequency, and the efficiency of the two sgRNAs used on two rice loci (*PDS* and *Chalk5*) was comparable. Furthermore, rate of point mutations in the two sites increased dramatically in the progeny, yet targeted deletions remained undetectable. Consistent with our study, others have also reported a much lower rate of genomic deletions by multiplex sgRNAs that is generally one order of magnitude lower than targeted point mutagenesis at two or more sites in the segment of the genome (Durr et al. 2018; Ordon et al. 2017).

At the outset, these observations suggest that multiplex targeting by CRISPR/Cas9 occurs through non-concurrent activity on different sites as a result of dissimilar sgRNA

efficiencies. Low rate of deletions in *GUS*, as observed in this study, could be based on dissimilar sg1 and sg2 efficiencies. However, genomic deletions in *PDS* and *Chalk5* that were targeted by equally efficient sgRNA pairs were not proportionately increased. Therefore, understanding of the kinetics of Cas9-generated DSB could lend a mechanistic explanation. The Cas9:sgRNA complex stays bound to the broken termini of the DNA (Jiang and Doudna 2017; Sternberg et al. 2014), which may prevent the free-fragment from being physically removed from the site. Subsequently, the free-fragment could participate in the NHEJ process and eventually be glued back to the genome. Thus, simultaneous DSBs end up with point mutations at each site rather than fragment deletion. Our dual-targeting data on three loci with highly variable efficiencies of sgRNA suggest that although sgRNA efficiency and Cas9 expression are important for the success of targeting, above a threshold, these parameters are unlikely to improve the rate of genomic deletions. Furthermore, DNA repair mechanisms in plants could affect the targeting outcome and enforce DSB repair by preserving broken termini and introducing only small indels, the most commonly observed effect of CRISPR/Cas9 targeting in plants (Mao et al. 2013). Nevertheless, heritability of genomic deletions and other editing effects could be improved by expressing Cas9 by germline promoters (Durr et al. 2018; Feng et al. 2018). Finally, the survey of mutations in multiple transformed lines obtained from different experiments showed that the same type of mutation occurred frequently in the DSB sites. While sg1 site mostly lost a nucleotide (−1), the sg2 site gained one (+1). The mechanistic explanation of this curious observation is not clear, but it implicates the role of target site and/or genomic context. More analysis with additional sgRNAs is needed to better understand the frequency of a given type of mutation in CRISPR/Cas9 targeting; however, similar observations have been made by Jacobs et al. (2015), who found identical mutation in multiple soybean lines. In a separate study based on targeting 10 loci in rice, +1 was found to be the most common mutation (> 50%), followed by −1 (Zhang et al. 2014). However, our data suggest that a target site could also have the preference for either an insertion (+1) or a deletion (−1).

In summary, consistent with a previous report on CRISPR/Cas9 targeting in rice (Jang et al. 2016), this study found that primary regenerated plants mostly harbor chimeric mutational effects. However, since the observed effects are generally not heritable, PCR screening at an early stage of callus growth, and isolation of the calli harboring the deletions will be an important step in recovering stable deletion lines. In addition, this study found that the types of mutations induced at a specific site by CRISPR/Cas9 are not highly variable, and frequently, the same type of mutation is observed from different targeting events. This observation

suggests that DSB repair is highly dependent on the target sequence.

Materials and methods

DNA constructs and plant transformation

The sgRNA spacer sequences were selected using CRISPR RGEN tool (<http://www.rgenome.net/cas-designer/>; Park et al. 2015). Vector pRGE32 (Addgene#63159) was used for synthesizing the CRISPR/Cas9-targeting vectors pJU24, pJU34, and pJU46 against *GUS* (NCBI accession no. AF485783), *OsPDS* (Os03g08570), and *Chalk5* (Chromosome 5: 3,335,405–3,341,600) genes, respectively. The two sgRNAs targeting each gene were expressed as polycistronic tRNA–gRNA (PTG) genes, which was synthesized against pGTR (Addgene# 63143) using the protocol of Xie et al. (2015). The constructed PTG (tRNA–gRNA1–tRNA–gRNA2) was ligated to pRGE32 vector by FokI/BsaI digestions, and the resulting vectors were used for rice transformations. The gRNA oligos used for PTG construction are given in Table 4. For targeting *GUS*, B1 transgenic line (cv. Nipponbare) was used for transformation as described earlier by Srivastava et al. (2017), while Nipponbare was used for targeting rice genes, *OsPDS* and *OsChalk5*. The embryogenic callus from mature seeds was used for all transformations by the gene gun (PDS1000, Bio-Rad Inc.), in which pJU24, pJU34, or pJU46 DNA was co-bombarded with hygromycin phospho-transferase expressing vector, p35S:HPT. The transformed calli were isolated and regenerated on hygromycin (50 mg/l) containing media using the protocol of Nishimura et al. (2006).

Molecular analysis

Genomic DNA isolated from callus, regenerated plants or seedlings, was used for polymerase chain reaction (PCR) using primers spanning the target sites (Table 4). The PCR products were resolved on agarose gel and extracted using GeneClean Spin Kit (MP Biomedicals, CA, USA) for sequencing from both ends using forward and reverse primers by the Sanger Sequencing method at Eurofins Genomics USA. The sequences were viewed on Sequence Scanner 2 software (Applied Biosystems Inc.) and aligned with the reference sequences using CLUSTAL-Omega multiple sequence alignment tool. CRISPR-ID tool was used to separate superimposed overlapping spectrum in Sanger sequencing traces, characteristic of heterozygous or chimeric mutations (Dehairs et al. 2016). The type of indel was identified by cloning PCR amplicon into pCR2.1 vector using TA cloning kit (Thermo-Fisher Scientific, NY) as per manufacturer's

Table 4 Primers used in the study

Primer	Sequence (5′–3′)	Application
gGus1F	TAGGTCTCCTGATCAGCGTTGGgttttagagctagaa	Construction of sgRNA1 (<i>GUS</i>): 5′-GTGGAATTGATCAGCGTTGG-3′
gGus1R	CGGGTCTCAATCAATTCCACtgcaccagccggg	
gGus2F	TAGGTCTCCCCGCAAACCGAAGTgttttagagctagaa	Construction of sgRNA2 (<i>GUS</i>): 5′-ACGCGACCGCAAACCGAA
gGus2R	CGGGTCTCAGCGGTTCGCGTtgcaccagccggg	GT-3′
gPDS1F	TAGGTCTCCAGGAGAATTCAGCgttttagagctagaa	Construction of sgRNA1 (<i>OsPDS</i>): 5′-ACAAGCCAGGAGAATTCA
gPDS1R	CGGGTCTCACCTGGCTTGTtgcaccagccggg	GC-3′
gPDS2F	TAGGTCTCCATGGATAACTCATCgttttagagctagaa	Construction of sgRNA2 (<i>OsPDS</i>): 5′-CACTGCATGGATAACTCA
gPDS2R	CGGGTCTCACCATGCAGTtgcaccagccggg	TC-3′
gChalk1F	TAGGTCTCCTCATGTAGATCTTgttttagagctagaa	Construction of sgRNA1 (<i>OsChalk5</i>): 5′-CTCTGGGTCATGTAGATC
gChalk1R	CGGGTCTCAATGACCCAGAGtgcaccagccggg	TT-3′
gChalk2F	TAGGTCTCCGACGGTGCCGTTTGTAGgttttagagctagaa	Construction of sgRNA2 (<i>OsChalk5</i>): 5′-GATTCTAGACGGTGCCGT
gChalk2R	CGGGTCTCAGCTCTAGAATCtgcaccagccggg	TTGTAG-3′
Ubi1812	TCTAACCTTGAGTACCTATCTATTA	Forward primer in B1 locus
NosR2	GCGGGACTCTAATCATAAAAACCC	Reverse primer in B1 locus
PDSF	GGTAGAAATGCCATGCGGGA	Forward primer in <i>OsPDS</i>
PDSR	GTGGTGAGGTTCCGGCTGAAT	Reverse primer in <i>OsPDS</i>
Chalk5F	ACAAGGCTAGCAAGTTGGC	Forward primer in <i>OsChalk5</i>
Chalk5R	CACTCGCTCGTCTTCTCCTC	Reverse primer in <i>OsChalk5</i>
Cas9F	AAAGACCGAGTGCAGACAG	Forward primer in Cas9
Cas9R	ACCAGCACAGAATAGGCCAC	Reverse primer in Cas9

instructions and sequencing individual colonies by Sanger sequencing.

Acknowledgements The vectors pRGE32, pRGE32, and pGTR were donated by Yinong Yang and obtained from Addgene.com. This project is supported by the Arkansas Division of Agriculture and USDA-NIFA Capacity Building Grant no. 2017-38821-26412 to MM and VS.

Author contributions BP did molecular analysis; SZ did rice transformations; MM participated in experimental design; VS and BP analyzed the data and wrote the manuscript.

Compliances with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Availability of data and materials The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

- Arsovski AA, Pradinuk J, Guo XQ, Wang S, Adams KL (2015) Evolution of cis-regulatory elements and regulatory networks in duplicated genes of Arabidopsis. *Plant Physiol* 169:2982–2991. <https://doi.org/10.1104/pp.15.00717>
- Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V (2013) Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Methods* 9:39. <https://doi.org/10.1186/1746-4811-9-39>
- Brooks C, Nekrasov V, Lippman ZB, Van Eck J (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 system. *Plant Physiol* 166:1292–1297. <https://doi.org/10.1104/pp.114.247577>
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339:819–823. <https://doi.org/10.1126/science.1231143>
- De Smet R, Van de Peer Y (2012) Redundancy and rewiring of genetic networks following genome-wide duplication events. *Curr Opin Plant Biol* 15:168–176. <https://doi.org/10.1016/j.pbi.2012.01.003>
- De Smet R, Sabaghian E, Li Z, Saeys Y, Van de Peer Y (2017) Coordinated functional divergence of duplicates in Arabidopsis. *Plant Cell* 29:2786–2800. <https://doi.org/10.1105/tpc.17.00531>
- Dehairs J, Talebi A, Cherifi Y, Swinnen JV (2016) CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing. *Sci Rep* 6:28973. <https://doi.org/10.1038/srep28973>
- Durr J, Papareddy R, Nakajima K, Gutierrez-Marcos J (2018) Highly efficient heritable targeted deletions of gene clusters and non-coding regulatory regions in Arabidopsis using CRISPR/Cas9. *Sci Rep* 8:4443. <https://doi.org/10.1038/s41598-018-22667-1>
- Falabella M, Sun L, Barr J, Pena AZ, Kershaw EE, Gingras S, Goncharova EA, Kaufman BA (2017) Single-step qPCR and dPCR

- detection of diverse CRISPR-Cas9 gene editing events in vivo. *G3* 7:3533–3542. <https://doi.org/10.1534/g3.117.300123>
- Feng C, Su H, Bai H, Wang R, Liu Y, Guo X, Liu C, Zhang J, Yuan J, Birchler JA, Han F (2018) High-efficiency genome editing using a dmc1 promoter-controlled CRISPR/Cas9 system in maize. *Plant Biotechnol J*. <https://doi.org/10.1111/pbi.12920>
- Gidoni D, Srivastava V, Carmi N (2008) Site-specific excisional recombination strategies for elimination of undesirable transgenes from crop plants. *In Vitro Cell. Dev Biol Plant* 44:457–467. <https://doi.org/10.1007/s11627-008-9140-3>
- Haberer G, Hindemitt T, Meyers BC, Mayer KF (2004) Transcriptional similarities, dissimilarities, and conservation of cis-elements in duplicated genes of Arabidopsis. *Plant Physiol* 136:3009–3022. <https://doi.org/10.1104/pp.104.046466>
- Huang X, Qian Q, Liu Z, Sun H, He S, Luo D, Xia G, Chu C, Li J, Fu X (2009) Natural variation at the DEP1 locus enhances grain yield in rice. *Nat Genet* 41:494–497. <https://doi.org/10.1038/ng.352>
- Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA (2015) Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnol* 15:16. <https://doi.org/10.1186/s12896-015-0131-2>
- Jang G, Lee S, Um TY, Chang SH, Lee HY, Chung PJ, Kim J-K, Choi YD (2016) Genetic chimerism of CRISPR/Cas9-mediated rice mutants. *Plant Biotechnol Rep* 10:425–435. <https://doi.org/10.1007/s11816-016-0414-7>
- Jasin M, Haber JE (2016) The democratization of gene editing: insights from site-specific cleavage and double-strand break repair. *DNA Repair* 44:6–16. <https://doi.org/10.1016/j.dnarep.2016.05.001>
- Jiang F, Doudna JA (2017) CRISPR–Cas9 structures and mechanisms. *Ann Rev Biophys* 46:505–529. <https://doi.org/10.1146/annurev-biophys-062215-010822>
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816–821. <https://doi.org/10.1126/science.1225829>
- Kapusi E, Corcuera-Gómez M, Melnik S, Stoger E (2017) Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in barley. *Front Plant Sci*. <https://doi.org/10.3389/fpls.2017.00540>
- Kim HJ, Lee HJ, Kim H, Cho SW, Kim JS (2009) Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res* 19:1279–1288. <https://doi.org/10.1101/gr.089417.108>
- Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *Plant J* 10:165–174. <https://doi.org/10.1046/j.1365-313X.1996.10010165.x>
- Lemos BR, Kaplan AC, Bae JE, Ferrazzoli AE, Kuo J, Anand RP, Waterman DP, Haber JE (2018) CRISPR/Cas9 cleavages in budding yeast reveal templated insertions and strand-specific insertion/deletion profiles. *Natl Acad Sci, Proc*. <https://doi.org/10.1073/pnas.1716855115>
- Liu S-L, Baute GJ, Adams KL (2011) Organ and cell type-specific complementary expression patterns and regulatory neofunctionalization between duplicated genes in *Arabidopsis thaliana*. *Genome Biol Evol* 3:1419–1436. <https://doi.org/10.1093/gbe/evr114>
- Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA guided human genome engineering via Cas9. *Science* 339:823–826. <https://doi.org/10.1126/science.1232033>
- Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu J-K (2013) Application of the CRISPR–Cas system for efficient genome engineering in plants. *Mol Plant* 6:2008–2011. <https://doi.org/10.1093/mp/sst121>
- Minkenberg B, Xie K, Yang Y (2017) Discovery of rice essential genes by characterizing a CRISPR-edited mutation of closely related rice MAP kinase genes. *Plant J* 89:636–648. <https://doi.org/10.1111/tpj.13399>
- Mojica FJ, Díez-Villaseñor C, García-Martínez J, Almendros C (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155:733–740. <https://doi.org/10.1099/mic.0.023960-0>
- Nandy S, Srivastava V (2012) Marker-free site-specific gene integration in rice based on the use of two recombination systems. *Plant Biotechnol J* 10:904–912. <https://doi.org/10.1111/j.1467-7652.2012.00715.x>
- Nekrasov V, Wang C, Win J, Lanz C, Weigel D, Kamoun S (2017) Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Sci Rep* 7:482. <https://doi.org/10.1038/s41598-017-00578-x>
- Nishimura A, Aichi I, Matsuoka M (2006) A protocol for Agrobacterium mediated transformation in rice. *Nat Protoc* 1:2796–2802. <https://doi.org/10.1038/nprot.2006.469>
- Ordon J, Gantner J, Kemna J, Schwalgun L, Reschke M, Streubel J, Boch J, Stuttmann J (2017) Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome editing toolkit. *Plant J*. <https://doi.org/10.1111/tpj.13319>
- Park J, Bae S, Kim J-S (2015) Cas-Designer: a web-based tool for choice of CRISPR-Cas9 target sites. *Bioinformatics* 31:4014–4016. <https://doi.org/10.1093/bioinformatics/btv537>
- Puchta H, Dujon B, Hohn B (1996) Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. *Proc Natl Acad Sci USA* 93:5055–5060. <https://doi.org/10.1073/pnas.93.10.5055>
- Rouet P, Smih F, Jasin M (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol* 14:8096–8106
- Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M, Hakimi SM, Mo H, Habben JE (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnol J* 15:207–216. <https://doi.org/10.1111/pbi.12603>
- Soltis DE, Visger CJ, Soltis PS (2014) The polyploidy revolution then...and now: stebbins revisited. *Am J Bot* 101:1057–1078. <https://doi.org/10.3732/ajb.1400178>
- Srivastava V, Underwood JL, Zhao S (2017) Dual-targeting by CRISPR/Cas9 for precise excision of transgene from rice genome. *Plant Cell Tissue Organ Cult* 129:153–160. <https://doi.org/10.1007/s11240-016-1166-3>
- Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 507:62–67. <https://doi.org/10.1038/nature13011>
- Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW (1983) The double-strand-break repair model for recombination. *Cell* 33:25–35
- Tian S, Jiang L, Gao Q, Zhang J, Zong M, Zhang H, Ren Y, Guo S, Gong G, Liu F, Xu Y (2017) Efficient CRISPR/Cas9-based gene knockout in watermelon. *Plant Cell Rep* 36:399. <https://doi.org/10.1007/s00299-016-2089-5>
- van Overbeek M, Capurso D, Carter MM, Thompson MS, Frias E, Russ C, Reece-Hoyes JS, Nye C, Gradia S, Vidal B, Zheng J, Hoffman GR, Fuller CK, May AP (2016) DNA repair profiling reveals non-random outcomes at Cas9-mediated breaks. *Mol Cell* 63:633–646. <https://doi.org/10.1016/j.molcel.2016.06.037>
- Voytas DF (2013) Plant genome engineering with sequence-specific nucleases. *Annu Rev Plant Biol* 64:327–350. <https://doi.org/10.1146/annurev-arplant-042811-105552>
- Wallace BD, Wang H, Lane KT, Scott JE, Orans J, Koo JS, Venkatesh M, Jobin C, Yeh LA, Mani S, Redinbo MR (2010) Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. *Science* 330:831–835. <https://doi.org/10.1126/science.1191175>

- Wang Y, Yau Y-Y, Perkins-Balding D, Thomson JG (2011) Recombinase technology: applications and possibilities. *Plant Cell Rep* 30:267–285. <https://doi.org/10.1007/s00299-010-0938-1>
- Wang Y, Geng L, Yuan M, Wei J, Jin C, Li M, Yu K, Zhang Y, Jin H, Wang E, Chai Z (2017a) Deletion of a target gene in Indica rice via CRISPR/Cas9. *Plant Cell Rep* 36:1333–1343. <https://doi.org/10.1007/s00299-017-2158-4>
- Wang X, Tu M, Wang D, Liu J, Li Y, Li Z, Wang Y, Wang X (2017b) CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. *Plant Biotechnol J* 16:844–855. <https://doi.org/10.1111/pbi.12832>
- Wang Z, Wang S, Li D, Zhang Q, Li L, Zhong C, Liu Y, Huang H (2018) Optimized paired-sgRNA/Cas9 cloning and expression cassette triggers high-efficiency multiplex genome editing in kiwifruit. *Plant Biotechnol J*. <https://doi.org/10.1111/pbi.12884>
- Waterworth WM, Drury GE, Bray CM, West CE (2011) Repairing breaks in the plant genome: the importance of keeping it together. *New Phytol* 192:805–822. <https://doi.org/10.1111/j.1469-8137.2011.03926.x>
- Wessler S, Tarpley A, Purugganan M, Spell M, Okagaki R (1990) Filler DNA is associated with spontaneous deletions in maize. *Proc Natl Acad Sci USA* 87:8731–8735
- Xie K, Yang Y (2013) RNA-guided genome editing in plants using a CRISPR-Cas system. *Mol Plant* 6:1975–1983. <https://doi.org/10.1093/mp/sst119>
- Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc Natl Acad Sci USA* 112:3570–3575. <https://doi.org/10.1073/pnas.1420294112>
- Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N, Zhu JK (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol J* 12(6):797–807. <https://doi.org/10.1111/pbi.12200>
- Zhou H, Liu B, Weeks DP, Spalding MH, Yang B (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucl Acids Res* 42:10903–10914. <https://doi.org/10.1093/nar/gku806>