SCIENCE CHINA
Life Sciences

SPECIAL TOPIC: Genome editing in genetic therapy and agriculture
• RESEARCH PAPER •

May 2017 Vol.60 No.5: 506–515
doi: 10.1007/s11427-017-9008-8

Rapid generation of genetic diversity by multiplex CRISPR/Cas9 genome editing in rice

Lan Shen[1,2†], Yufeng Hua[1†], Yaping Fu[1†], Jian Li[3†], Qing Liu[1], Xiaozhen Jiao[1], Gaowei Xin[4], Junjie Wang[1], Xingchun Wang[4], Changjie Yan[2*] & Kejian Wang[1*]

[1]*State Key Laboratory of Rice Biology, China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou 310006, China;*
[2]*Key Laboratory of Plant Functional Genomics, Ministry of Education, Yangzhou University, Yangzhou 225009, China;*
[3]*Lianyungang Academy of Agricultural Sciences, Lianyungang 222006, China;*
[4]*College of Life Sciences, Shanxi Agricultural University, Taigu 030801, China*

Received December 1, 2016; accepted December 20, 2016; published online March 24, 2017

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease 9 (CRISPR/Cas9) system has emerged as a promising technology for specific genome editing in many species. Here we constructed one vector targeting eight agronomic genes in rice using the CRISPR/Cas9 multiplex genome editing system. By subsequent genetic transformation and DNA sequencing, we found that the eight target genes have high mutation efficiencies in the T_0 generation. Both heterozygous and homozygous mutations of all editing genes were obtained in T_0 plants. In addition, homozygous sextuple, septuple, and octuple mutants were identified. As the abundant genotypes in T_0 transgenic plants, various phenotypes related to the editing genes were observed. The findings demonstrate the potential of the CRISPR/Cas9 system for rapid introduction of genetic diversity during crop breeding.

CRISPR/Cas9, genome editing, agronomic genes, rice

Citation: Shen, L., Hua, Y., Fu, Y., Li, J., Liu, Q., Jiao, X., Xin, G., Wang, J., Wang, X., Yan, C., and Wang, K. (2017). Rapid generation of genetic diversity by multiplex CRISPR/Cas9 genome editing in rice. Sci China Life Sci 60, 506–515. doi: 10.1007/s11427-017-9008-8

INTRODUCTION

Mutants are crucial for studying gene function and crop breeding. The traditional artificial mutations are usually created by ethyl methanesulfonate mutagenesis, irradiation mutagenesis, and transfer DNA insertion. However, these methods cause random mutagenesis in the genome, thereby necessitating laborious and time-consuming identification of mutated genes for further study.

Over the past decade, new technologies collectively referred to as genome editing have emerged (Li et al., 2011; Wood et al., 2011; Shan et al., 2013), which include the zinc finger nuclease technology, transcription activator-like effector nuclease technique, and type II clustered regularly interspaced short palindromic repeat (CRISPR)-associated endonuclease 9 (CRISPR/Cas9) systems. Of these, the CRISPR/Cas9 system, which is developed from the microbial adaptive immune system, is the most widely used. This system has two crucial components: a Cas9 protein and a single-stranded guide RNA (sgRNA), which is designed by fusing a CRISPR RNA (crRNA) and a trans-activat-

†Contributed equally to this work
*Corresponding author (Changjie Yan, email: cjyan@yzu.edu.cn; Kejian Wang, email: wangkejian@caas.cn)

© Science China Press and Springer-Verlag Berlin Heidelberg 2017

ing crRNA. The sgRNA is used to recognize alternative DNA sequences with a short protospacer adjacent motif based on base pairing and then guiding the Cas9 nuclease to cleave the DNA sequences of interest. The DNA double-strand breaks due to the cleavage caused by Cas9 nuclease can induce various mutations via the error-prone non-homologous end-joining pathway or the homology-directed repair pathway. Mutations, such as base insertions, deletions, and substitutions may induce frame shift, resulting in loss-of-function mutants.

The generation of multiple gene mutations is required for investigating the relationship among several related genes and the function of gene families (Ma et al., 2015b; Li et al., 2016; Shen et al., 2016; Zhang et al., 2016). To date, various multiplex CRISPR/Cas9 systems have been developed in many organisms (Generoso et al., 2016; Liu et al., 2016; Ma and Liu, 2016; Ma et al., 2016; Qi et al., 2016; Sakuma et al., 2016; Wang et al., 2016). These systems use different strategies to integrate multiple sgRNA and Cas9 expression cassettes into a single binary vector. The most popular strategy used for assembling the vector relies on the Golden Gate ligation or Gibson assembly methods (Patron, 2014; Xing et al., 2014; Casini et al., 2015; Ma et al., 2015b). Previously, we developed a simple system for multiplex genome editing using the classical isocaudamer technique (Wang et al., 2015a). This simple system can theoretically be used for simultaneous editing of an unlimited number of genes.

With the availability of rice genome sequence, many agronomic genes have been identified in rice. In the present study, we aimed to modify eight agronomic genes using the CRISPR/Cas9 multiplex genome editing system in rice. Our results show that eight agronomic genes can be simultaneously modified in one generation by a single transformation in rice. In addition, mutants with various gene combinations were simultaneously obtained. As generating population diversity for selection is one of the main pursuits during crop breeding, the results provide a strategy trial for rapid generation of various genetic materials using the CRISPR/Cas9 system.

RESULTS

CRISPR/Cas9 multiplex-genome editing system for eight gene targeting in rice

Previously, we performed CRSIPR/Cas9-mediated QTL editing and unexpectedly found that the same QTLs can have highly varied, even opposing, yield performance in different rice varieties (Shen et al., 2016). We therefore randomly selected eight agronomic genes for the present study, namely *BADH2* (betaine aldehyde dehydrogenase 2), *DEP1* (dense and erect panicle 1), *Gn1a* (grain number), *QTL* (major quantitative trait loci), *GS3* (for grain length and weight), *GW2* (major *QTL* associated with rice grain width and weight), *Hd1* (heading date 1), *EP3* (*ERECT PANICLE*), and *LPA1* (loose plant architecture 1). Five of these eight genes (*DEP1*, *EP3*, *Gn1a*, *GS3*, and *GW2*) are related to rice yield, one gene (*LPA1*) regulates the plant architecture, and *BADH2* and *Hd1* have a relationship with rice fragrance and photoperiod, respectively.

To improve plant panicles, we selected three panicle-related genes, namely *DEP1*, *EP3*, and *Gn1a*, for genome editing. Previous research revealed that mutations in *DEP1* and *EP3* show improved panicle architecture (Huang et al., 2009; Piao et al., 2009; Zhou et al., 2009; Yu et al., 2015), and mutation of *Gn1a* led to an increased grain number per panicle (Ashikari et al., 2005). We used the CRISPR/cas9 system to target *DEP1* (15–36 bp in the first exon in complementary DNA (cDNA)), *EP3* (261–282 bp in the first exon in cDNA), and *Gn1a* (322–343 bp in the first exon in cDNA) (Figure 1A–C, respectively). Besides the plant panicles, the grain size is also an important component for grain yield. In the present study, we examined two widely used QTLs, *GS3* and *GW2*. *GS3* mutants display longer grain length (Fan et al., 2006; Mao et al., 2010), and the mutants of *GW2* show wider grain size and enhanced 1,000-grain weight (Song et al., 2007); therefore, *GS3* and *GW2* were chosen for creating longer and wider seed size. We selected the targeting sequence starting from 97 to 118 bp from the ATG codon in the genomic DNA for editing *GS3* (Figure 1D) and the target sequence in the first exon (600–621 bp from the ATG codon in the cDNA) for targeting *GW2* (Figure 1E). Plant architecture is an important phenotypic parameter for developing high yielding rice varieties. We selected the plant type-related gene *LPA1*; the loss-of-function mutants of *LPA1* exhibit loose plant architecture (Wu et al., 2013). We targeted the sequence from 319 to 400 bp from the ATG codon in the second exon (Figure 1F). To obtain the mutants with fragrance and controlled growth period, we destroyed the other two genes, *BADH2* and *Hd1* (Yano et al., 2000; Chen et al., 2008). The target site of *BADH2* contains the ATG codon in the genomic DNA (Figure 1G) and that of *Hd1* is located in the first exon (251–272 bp from the ATG codon) (Figure 1H).

The technological process to construct the CRISPR/Cas9 vector for targeting eight genes simultaneously was based on the conventional isocaudamer-based method (Figure 2). Firstly, the eight target sequences were respectively cloned to the intermediate vector called SK-gRNA. We assembled four gRNAs into one intermediate vector (assemble SK-gRNA-*BADH2*, SK-gRNA-*LPA1*, SK-gRNA-*GS3* and SK-gRNA-*Hd1* to SK-gRNA-*BADH2*-*LPA1*-*GS3*-*Hd1*; assemble SK-gRNA-*DEP1*, SK-gRNA-*Gn1a*, SK-gRNA-*GW2*, and SK-gRNA-*EP3* to SK-gRNA-*DEP1*-*Gn1a*-*GW2*-*EP3*). Subsequently, we assembled the two intermediate vectors (SK-gRNA-*BADH2*-*LPA1*-*GS3*-*Hd1* and SK-gRNA-

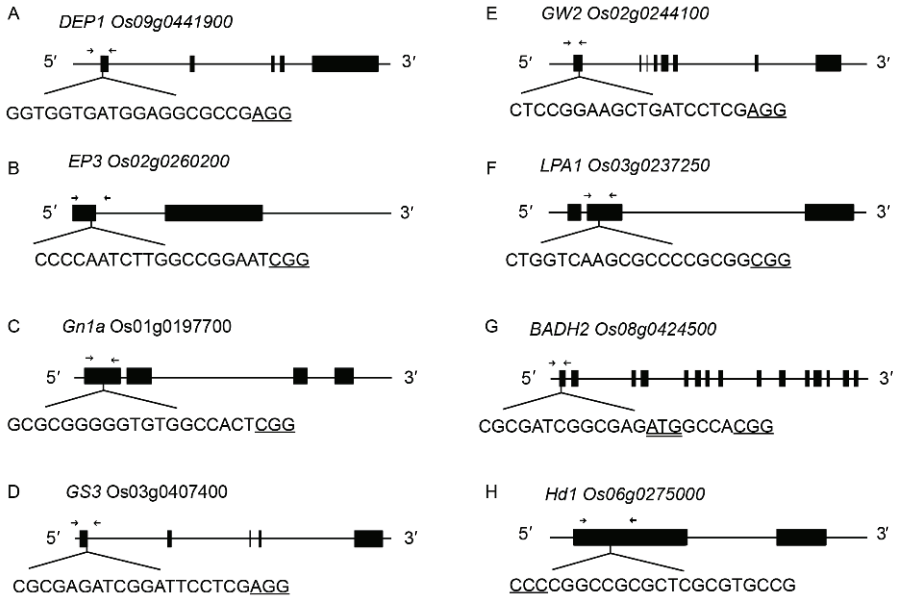

Figure 1 Schematic diagram of the targeted sites in eight genes. A–H, The targeted sites are labeled in black uppercase letters. The initiation codons are underlined twice. The protospacer adjacent motif (PAM) sequences are underlined once. The arrows show the regions around the editing sites.

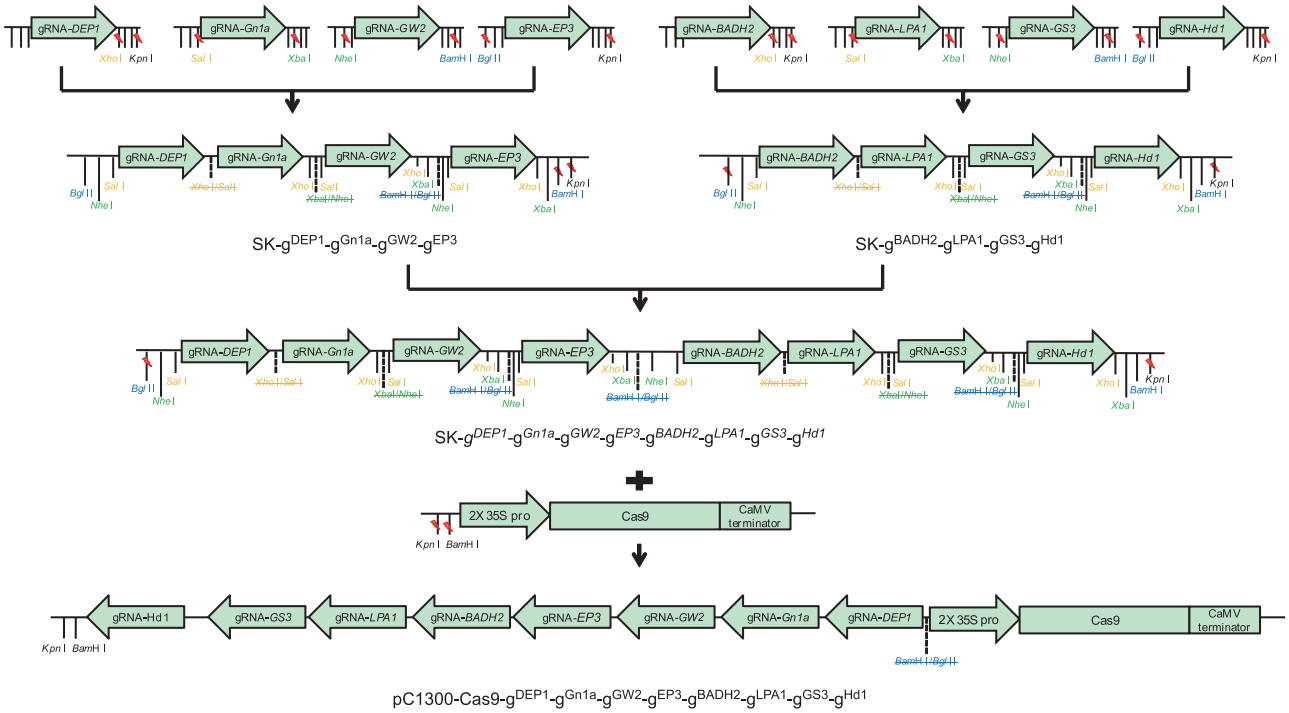

Figure 2 Flow diagram of an octuple CRISPR/Cas9 system for multiplex gene editing in rice. The restriction sites used for cloning are labeled. *Bam*H I+*Bgl* II, *Nhe* I+*Xba* I, and *Sal* I+*Xho* I are isocaudamer pairs and are highlighted in blue, green, and yellow, respectively.

DEP1-Gn1a-GW2-EP3) into one intermediate vector with eight target sites. Finally, the eight sgRNA modules were digested with the restriction enzymes for cloning into the pC1300-Cas9 expression vector.

Editing efficiency of single target in rice protoplast

To determine whether the vectors have a function in editing multiple genes using the CRISPR/Cas9 system, we co-transformed the sgRNAs and Cas9 protein using the transient expression system in rice protoplast and found through the polymerase chain reaction (PCR)/restriction endonuclease (RE) assay that all eight targeting sites were modified in rice protoplast (Figure 3). By comparing the mutation efficiency at the *BADH2* target site in three independent vectors (SK-

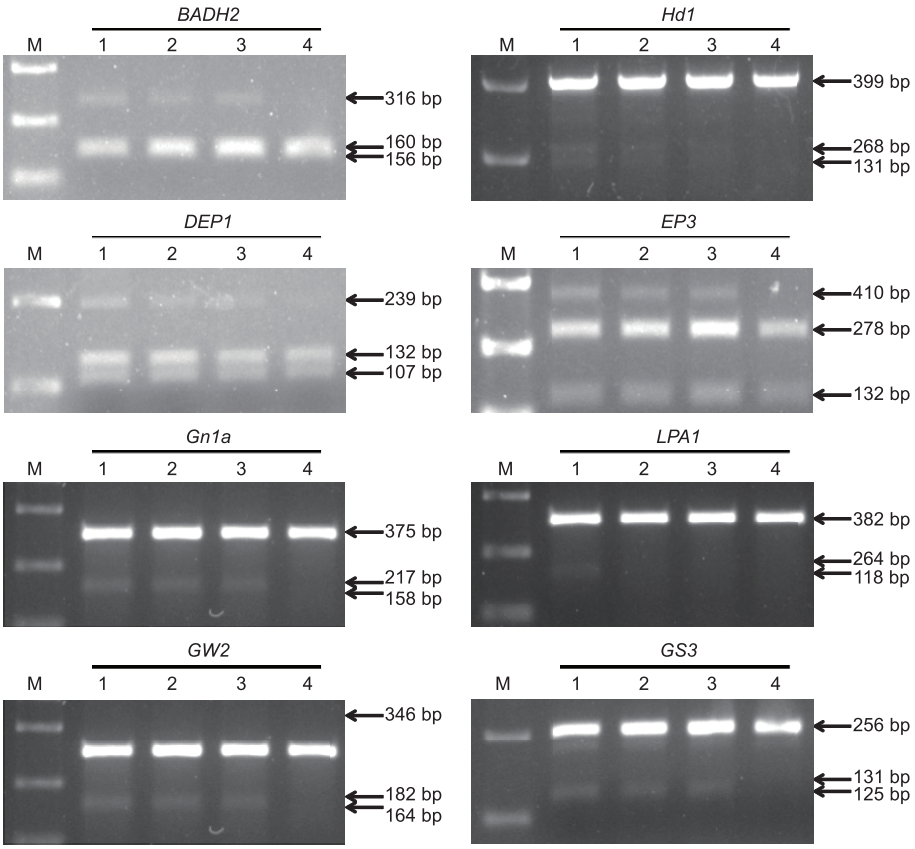

Figure 3 PCR/RE assay of mutations at eight loci in rice protoplast. *BADH2*, *DEP1*, *Gn1a*, *GW2*, *Hd1*, *EP3*, *LPA1*, and *GS3* products were digested with *MscI*, *NarI*, *MscI*, *T7E1*, *T7E1*, *HinfI*, *T7EI*, and *T7EI*, respectively. Lane M, DNA marker. Lane 1, results of PCR/RE assay co-transformed in the single sgRNA (SK-gRNA-*BADH2* SK-gRNA-*DEP1* SK-gRNA-*Gn1a* SK-gRNA-*GW2* SK-gRNA-*Hd1* SK-gRNA-*EP3* SK-gRNA-*LPA1*, and SK-gRNA-*GS3*, respectively) and Cas9 protein using the transient expression system in rice protoplast. Lane 2, results of PCR/RE assay co-transformed in the four sgRNAs (SK-gRNA-*DEP1-Gn1a-GW2-EP3* and SK-gRNA-*BADH2-LPA1-GS3-Hd1*, respectively) and Cas9 protein using the transient expression system in rice protoplast. Lane 3, results of PCR/RE assay co-transformed in the eight sgRNAs (SK-gRNA-*DEP1-Gn1a-GW2-EP3-BADH2-LPA-GS3-Hd1*) and Cas9 protein using the transient expression system in rice protoplast. Lane 4, results of PCR/RE assay co-transformed in the control sgRNA (SK-gRNA) and Cas9 protein by using the transient expression system in rice protoplast.

gRNA-*BADH2*, SK-gRNA-*BADH2-LPA1-GS3-Hd1*, and SK-gRNA-*DEP1-Gn1a-GW2-EP3-BADH2-LPA1-GS3-Hd1*), we found that the *BADH2* mutation efficiency is almost the same in single gene knockout and multiplex genes knockout by the transient expression system, and similar results were obtained for the other seven genes (Figure 3), indicating that the cascade of sgRNAs might not affect the mutation rate of CRISPR/Cas9.

Characterization of targeted editing in T_0 rice plants

The binary vector targeting eight genes was used for genetic transformation. A total of 36 positive transgenic plants were obtained in the T_0 generation. To analyze the mutations of targeting sites, we sequenced eight target regions in all positive transgenic plants. The mutagenesis efficiencies of the eight targeting sites were 50%, 100%, 67%, 81%, 83%, 97%, 67%, and 78% for *EP3, GS3*, *GW2*, *BADH2, DEP1*, *Gn1a*, *LPA*1, and *Hd1*, respectively (Figure 4A). Moreover, we analyzed the genotype in each target site and found that, besides *GS3*, all the remaining seven genes have three genotypes, namely homozygous mutation (including biallelic mutations), heterozygous mutation, and the wild type (WT) (Figure 4B). We also found that the homozygous mutation efficiencies of four genes (*BADH2*, *Gn1a*, *GS3*, and *GW2*) were >55%. Moreover, the heterozygous mutation efficiencies of eight genes were <35% (Figure 4B). The varied mutation frequency and mutation types at eight target loci led to differed genotypes among transgenic plants.

In many cases, the generation of mutants containing different gene combinations is also required for genetic studies or crop breeding. To analyze the diversity of multiple genotypes in T_0 plants, we statistically analyzed the combination patterns of eight mutant genes. Of the 36 transgenic plants, a total of 25 different genotypes were found, including double mutations, quintuple mutations, sextuple mutations, septuple mutations, and octuple mutations (Table 1). Among these mutants, we detected four homozygous mutants (one homozygous sextuple mutant, one homozygous septuple mu-

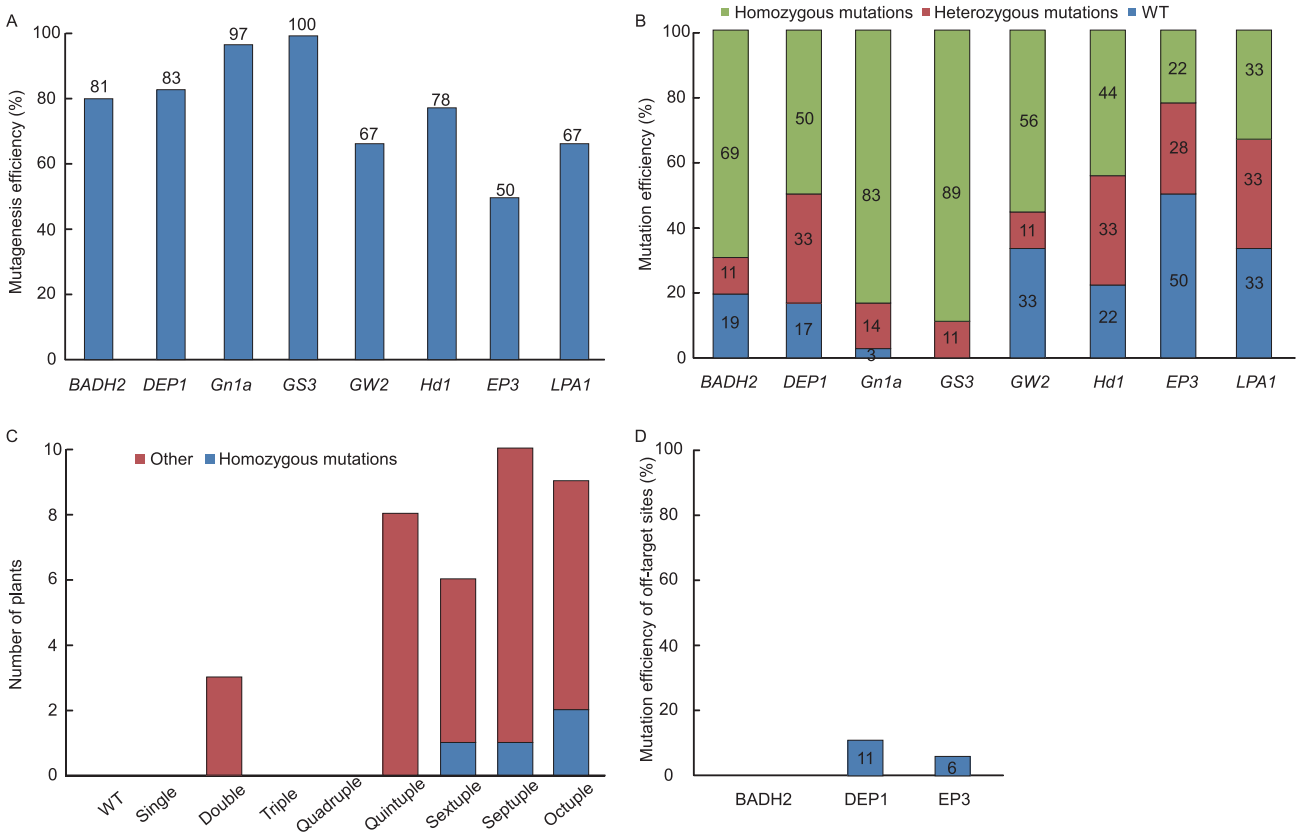

Figure 4 Characterization of targeted editing in T_0 rice plants. A, Editing efficiencies of eight agronomic genes in T_0 plants. B, Editing efficiencies of wild type (WT), homozygous mutations, and heterozygous mutations in each gene. C, Numbers of plants with different mutation genes. D, Editing efficiencies of off-target genes in T_0 plants.

Table 1 Combination patterns of eight agronomic genes[a)]

Type of gene mutation	Genotype	No. of plants	Sum
Double mutations	AABBccDdEEFFGGHH	3	3
Quintuple mutations	AabbccddEEffGGHH	2	8
	aaBbCcddEEffGGHH	1	
	aaBBccddEEFFGgHh	2	
	AABbccddEEffGgHH	1	
	AABbccddEEffGGHh	1	
	AABBCcDdeeffGGHh	1	
Sextuple mutations	aabbCcddeeffGGHH	1	6
	aaBbCcddeeffGGHH	2	
	aabbccddeeFfGGHH	1	
	aabbccddeeffGGHH	1	
	AaBbCCddEeFfGgHH	1	
Septuple mutations	aabbccddEEffGgHh	1	10
	aaBbccddEEFfGgHh	1	
	aabbccddeeFFGghh	2	
	aaBbccddEeFFGgHh	1	
	aabbccddeeFfGGhh	1	
	aabbccddeeffGGhh	1	
	aaBbccddeeFfGGhh	2	
	aabbccddeeffGGHh	1	
Octuple mutations	AaBbccddEeFfGgHh	1	9
	aabbccddeeFfGghh	4	
	aaBbccddEeFfGgHh	1	
	Aabbccddeeffgghh	2	
	AabbccddeeFfGgHh	1	

a) The letters (a–h), represent *BADH2*, *DEP1*, *Gn1a*, *GS3*, *GW2*, *Hd1*, *EP3*, *LPA1*, respectively.

tant, and two homozygous octuple mutants), and all the mutated genes contained homozygous mutations (Figure 4C). Therefore, one genetic transformation event resulted in many gene mutation combinations in T_0 generation plants.

Off-target events occurred at low frequency

Previous studies of many organisms revealed that off-target mutations usually occur during CRISPR/Cas9-mediated genome editing (Doench et al., 2016; Hu et al., 2016; Ma et al., 2016); therefore, we analyzed the eight target sites by National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST). We sequenced all three potential off-target sites (*BADH2*, *DEP1*, and *EP3*), and found that the rates of mutation were 0%, 11.1%, and 5.6%, respectively, which were much lower than those of the normal eight target sites (Figure 4D and Table S1 in Supporting Information).

Identification and phenotypic analysis of various mutants generated by targeting multiplex genes

Using the CRISPR/Cas9 system, we obtained various combinations of gene knockout mutants. To investigate whether the simultaneous multiplex gene mutants have the expected functions, we examined these homozygous mutations combined with the genetic phenotypes in the T_0 generation.

Previous studies showed that plants with the natural variation in the fifth exon induced the premature stop in *DEP1,* resulting in erect panicles with a shorter panicle length and increased grain number per panicle (Huang et al., 2009; Zhou et al., 2009). The loss of function mutants of *EP3* exhibited an increased number of inflorescence branches and increased grain number per panicle (Piao et al., 2009; Yu et al., 2015). The mutation of *Gn1a* resulted in enhanced grain yield by increasing the grain number per panicle (Ashikari et al., 2005). By sequencing the mutated regions of all 36 positive transgenic plants, we identified various mutations, including insertions, deletions, and substitutions (Figure S1 in Supporting Information). We observed the panicles of mutants with frame shift mutations, with the results showing that the panicle length of the *DEP1* and *Gn1a* co-mutation mutant (mutant-1) showed an erect panicle and marginally decreased grain number, whereas the mutant-2 with modified *Gn1a* showed an increased grain number (Figure 5).

Grain size-related gene *GS3*, which is a major QTL for grain length and grain weight, was edited in the present study. Mutants with the edited organ size regulation domain of *GS3* showed the longer grain length during previous studies (Fan et al., 2006; Mao et al., 2010). Compared with the WT, the *GS3* mutants had a significant increase in grain length (>8 mm) and enhanced grain weight (Figure 6A, C and E). The grain size is also influenced by an additional major QTL, *GW2*, which controls rice grain width and weight by negatively regulating cell division. A previous study showed that the loss-of-function mutants have increased grain width, increased grain weight, and enhanced yield (Song et al., 2007). We found that mutation of the *GW2* gene had wider seed size and increased 1,000-grain weight (Figure 6B, D and E), similar to the natural mutants.

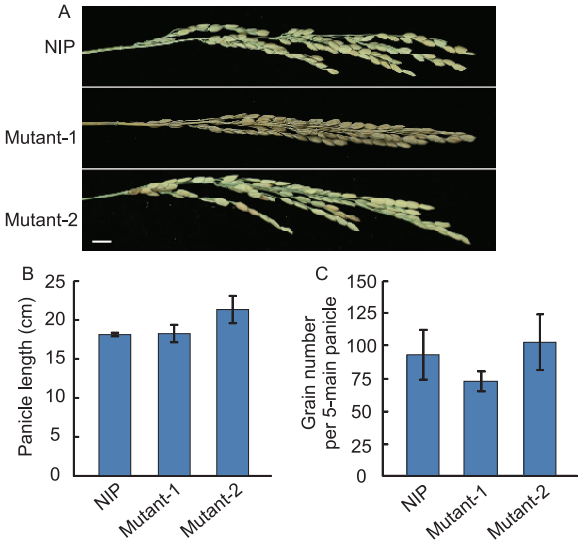

Figure 5 Comparison of panicle traits among Nipponbare (NIP), Mutant 1, and Mutant 2. A, The morphology of the panicles of the NIP, Mutant 1, and Mutant 2. Scale bar, 1 cm. B, Comparison of panicle length among NIP, Mutant 1, and Mutant 2. C, Comparison of grain number per panicle among NIP, Mutant 1, and Mutant 2. Values in B and C are means±standard deviations (SD), n=5. The genotype of mutant-1 and mutant-2 are aabbccddeeffgghh and AABBccDdEEFFGGHH. The letters (a–h), represent *BADH2*, *DEP1*, *Gn1a*, *GS3*, *GW2*, *Hd1*, *EP3*, *LPA1*, respectively.

We examined the rice gene *LPA1*, which determines plant architecture by regulating tiller and leaf angle (Wu et al., 2013). We found that plants with modified *LPA1* in our study exhibited exaggerated tiller angles and lamina angles (Figure 7), which is similar to the loose plant architecture phenotype observed in a previous study.

In addition to these genes related to rice yield, we also examined two other genes, *BADH2* and *Hd1*. The grains of mutants with destroyed *BADH2* have a characteristic fragrance (Chen et al., 2008). In addition, the modified QTL gene *Hd1* (response to photoperiod) should have delayed heading date under short-day conditions in previous studies (Yano et al., 2000). We detected the mutants by smelling the treated leaves, which is a very easy method reported previously (Sood and Sidiq, 1978). The detected leaves with fragrance were from the loss-of-function mutants, broadly in line with what we expected. Because the mutated plants were not handled with a different photoperiod, the heading date of mutants showed no difference from the WT.

DISCUSSION

The CRISPR/Cas9 system has been widely used in genome

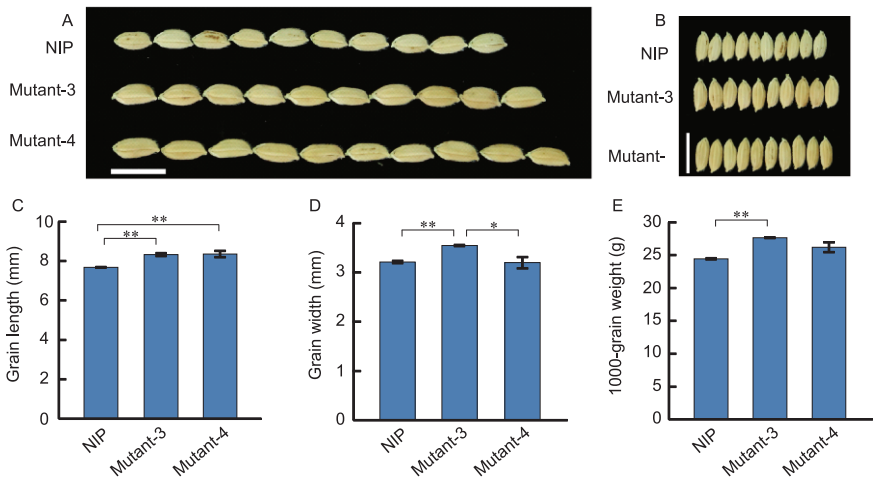

Figure 6 Comparison of seed size among Nipponbare (NIP), Mutant 3, and Mutant 4. Aand B, Grain shape of the NIP, Mutant 3, and Mutant 4. Scale bar, 1 cm. C, Comparison of grain length among NIP, Mutant 3, and Mutant 4. D, Domparison of grain width among NIP, Mutant 3, and Mutant 4. E, Comparison of 1,000-grain weight among NIP, Mutant 3, and Mutant 4. Values in C, D, and F are means±standard deviation (s.d.), n=20, and three replicates. The genotype of mutant-3 and mutant-4 are aabbccddeeffgghh and aaBBccddEEFFGgHh. The letters (a–h), represent *BADH2*, *DEP1*, *Gn1a*, *GS3*, *GW2*, *Hd1*, *EP3*, *LPA1*, respectively.

editing in many species due to its simplicity and high efficiency (Ma et al., 2015b; Generoso et al., 2016; Ma et al., 2016; Qi et al., 2016; Sakuma et al., 2016). In the present study, we showed that the CRISPR/Cas9 system can be successfully used to edit simultaneously eight agronomic genes by a single genetic transformation. Previous studies have reported that the repeat sequence could induce recombination of genes, which would result in gene silence (Fukuma et al., 2016; Mitsuda and Shimizu, 2016). In our research, the eight SK-gRNAs had the same promoter and structure sequences. However, we observed high mutation efficiencies at all eight genes in the T_0 generation. In addition, we detected mutations in eight genes in the transient expression system. Both results indicate that recombination between sgRNAs, if present, might not be sufficiently high to affect the implication of the CRISPR/Cas9 system.

Mutants are important for studying genes related to crop breeding. To date, a number of agronomic genes have been reported in different rice varieties and some have been widely used in rice breeding (Cho et al., 1994; Monna et al., 2002; Spielmeyer et al., 2002; Ashikari et al., 2005; Fan et al., 2006; Song et al., 2007; Miura et al., 2010; Jiang et al., 2013; Wang et al., 2015b; Si et al., 2016). However, the consolidation of several optimal genes from different rice varieties into one improved variety could pose a challenge. In the present study, the three panicle-related genes (*DEP1*, *EP3*, and *Gn1a*), two grain size related genes (*GS3* and *GW2*), one plant architecture-related gene (*LPA1*), one fragrance gene (*BADH2*), and a photoperiod-related gene (*Hd1*) were co-edited in the Nipponbare background using the CRISPR/Cas9 system. The three panicle-related genes commonly function to increase grain number per panicle, as previously reported, and the *dep1* and the *ep3* mutants display an erect panicle and larger panicle, respectively. We observed the phenotype of enhanced grain number in *gn1a* mutants, as expected. The *dep1* mutant in Nipponbare background displayed an erect panicle; however, the grain number was not increased and the setting percentage was low, probably caused by severe mutation of the *DEP1* gene in our study. For the grain size-regulating genes (*GS3*, *GW2*), we obtained mutants with larger grain size, in accordance with the natural mutants of *GS3* and *GW2*. We found that the *gs3* mutants had a longer grain length than that of the *GS3* and *GW2* co-mutation mutant, and the width of the *gs3* mutant was shorter than that of the *GS3* and *GW2* co-mutation mutant (Figure 7). The results show that *GW2* may have negative functions in the regulation of *GS3* function. For the plant architecture gene *LPA1*, the mutant in our study showed loose plant architecture similar to the phenotype reported before (Wu et al., 2013).

In conclusion, by a single genetic transformation, not only did we generate plants containing mutations in eight agronomic genes but also created mutants with various gene combinations in the T_0 generation. Moreover, both homozygous and heterozygous genotypes at eight genes were obtained. Thus, a population with higher phenotype diversity will be segregated out during the next generation. As generating population diversity for selection is one of the main pursuits during breeding, the work provides a strategy for rapid introduction of genetic diversity during crop breeding.

MATERIALS AND METHODS

Plant materials and growth conditions

The background of transgenic plants is Nipponbare and all the rice plants were grown in the paddy fields of the China

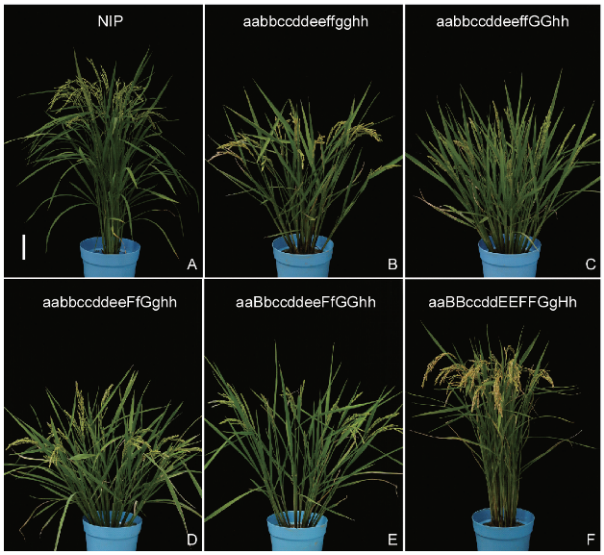

Figure 7 Phenotypes of plants with different gene combinations. A–F, Nipponbare (NIP). The letters (a–h), up from the plants represent *BADH2*, *DEP1*, *Gn1a*, *GS3*, *GW2*, *Hd1*, *EP3*, *LPA1*, respectively. Lowercase letters represent the modifed genes, whereas the uppercase letters indicate the normal genes. Scale bar, 10 cm.

National Rice Research Institute in Hangzhou, China during normal rice growing seasons.

Measurements of agronomic traits

Harvested rice was dried at 42°C for 2 days and stored at room temperature before testing. A total of 20 randomly chosen full filled seeds were used to measure grain length, grain width, and grain weight in the laboratory.

Plasmid construction

The eight target sites were designed for knock out of eight genes using the CRISPR/Cas9 system. The plasmid transferred into *Agrobacterium* was constructed by the isocaudamer ligation method, as previously described. The sgRNAs of *DEP1* (digested with *Kpn* I/*Xho* I), *Gn1a* (digested with *Sal* I/*Xba* I), *GW2* (digested with *Nhe* I/*Bam*H I), and *EP3* (digested with *Kpn* I/*Bgl* II) were assembled into one intermediate vector. Similar methods were used to assemble *BADH2*, *LPA1*, *GS3*, and *Hd1* into one intermediate vector. The two intermediate sgRNAs were assembled into one vector with eight sgRNAs, following which this intermediate vector (digested with *Kpn* I/*Bgl* II) was assembled to the pC1300-Cas9 binary vector (digested with *Kpn* I/*Bam*H I). The target sequences are provided in Table S2 in Supporting Information.

Agrobacterium-mediated rice transformation

The pC1300-Cas9 binary vector loading eight sgRNAs was used for genetic transformation via the *Agrobacterium*-mediated transformation (strain EHA105) method for generating transgenic rice, according to previously-described methods (Hiei et al., 1994).

Detection of mutations

Genomic DNA of transgenic plants was extracted from approximately 100 mg leaf tissue of rice via the cetyltrimethylammonium bromide (CTAB) method. PCR was conducted with KOD FX DNA polymerase (Toyobo, Japan) to amplify the fragments surrounding the eight target sites. The DNA fragments were sequenced by the Sanger method and analyzed by the degenerate sequence decoding method (Ma et al., 2015a).

Transient expression of the CRISPR/Cas9 system in rice protoplasts

Isolation of rice protoplasts was performed by the enzyme hydrolysis method (Shan et al., 2014). Transfection of the rice protoplast was performed by the polyethylene glycol-PEG-calcium method. A total of 20 μg total plasmids were used for each reaction. The transfected rice protoplasts were incubated in the dark at 28°C for 48 h.

PCR/RE assays

Genomic DNA of rice protoplasts was extracted by the CTAB method. PCR amplification of DNA sequences around the targeting sites was performed using KOD FX DNA Polymerase. PCR products were then digested by the appropriate restriction enzymes to analyze the mutagenesis efficiency.

Fragrance determination

Fragrance determination of rice leaves was conducted according to previously described methods (Sood and Sidiq, 1978). Approximately 0.5 g green leaves of rice tillering stage were sliced into sections and placed into 2 mL Eppendorf tubes. Each tube was incubated with 1.7% KOH solution at room temperature for 10 min, following which at least three people were asked to grade the fragrance by smelling.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

Acknowledgements *This work was supported by the National Natural Science Foundation of China (31271681, 3140101312), the Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences, and Jiangsu Agriculture Science and Technology Innovation Fund (CX(13)5075).*

Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., Angeles, E.R., Qian, Q., Kitano, H., and Matsuoka, M. (2005). Cytokinin oxidase regulates rice grain production. Science 309, 741–745.

Casini, A., Storch, M., Baldwin, G.S., and Ellis, T. (2015). Bricks and blueprints: methods and standards for DNA assembly. Nat Rev Mol Cell Biol 16, 568–576.

Chen, S., Yang, Y., Shi, W., Ji, Q., He, F., Zhang, Z., Cheng, Z., Liu, X., and Xu, M. (2008). *Badh2*, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. Plant Cell 20, 1850–1861.

Cho, Y.G., Eun, M.Y., McCouch, S.R., and Chae, Y.A. (1994). The semidwarf gene, *sd-1*, of rice (*Oryza sativa* L.). II. molecular mapping and

marker-assisted selection. Theoret Appl Genet 89, 54.
Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H.W., Listgarten, J., and Root, D.E. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol 34, 184–191.
Fan, C., Xing, Y., Mao, H., Lu, T., Han, B., Xu, C., Li, X., and Zhang, Q. (2006). GS3, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. Theor Appl Genet 112, 1164–1171.
Fukuma, M., Ganmyo, Y., Miura, O., Ohyama, T., and Shimizu, N. (2016). Cloning and characterization of a human genomic sequence that alleviates repeat-induced gene silencing. PLoS One 11, e0153338.
Generoso, W.C., Gottardi, M., Oreb, M., and Boles, E. (2016). Simplified CRISPR-Cas genome editing for *Saccharomyces cerevisiae*. J Microbiol Methods 127, 203–205.
Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6, 271–282.
Hu, X., Wang, C., Fu, Y., Liu, Q., Jiao, X., and Wang, K. (2016). Expanding the range of CRISPR/Cas9 genome editing in rice. Mol Plant 9, 943–945.
Huang, X., Qian, Q., Liu, Z., Sun, H., He, S., Luo, D., Xia, G., Chu, C., Li, J., and Fu, X. (2009). Natural variation at the *DEP1* locus enhances grain yield in rice. Nat Genet 41, 494–497.
Jiang, L., Liu, X., Xiong, G., Liu, H., Chen, F., Wang, L., Meng, X., Liu, G., Yu, H., Yuan, Y., Yi, W., Zhao, L., Ma, H., He, Y., Wu, Z., Melcher, K., Qian, Q., Xu, H.E., Wang, Y., and Li, J. (2013). DWARF 53 acts as a repressor of strigolactone signalling in rice. Nature 504, 401–405.
Li, M., Li, X., Zhou, Z., Wu, P., Fang, M., Pan, X., Lin, Q., Luo, W., Wu, G., and Li, H. (2016). Reassessment of the four yield-related genes *Gn1a*, *DEP1*, *GS3*, and *IPA1* in rice using a CRISPR/Cas9 system. Front Plant Sci 7, 377.
Li, T., Huang, S., Zhao, X., Wright, D.A., Carpenter, S., Spalding, M.H., Weeks, D.P., and Yang, B. (2011). Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Res 39, 6315–6325.
Liu, X., Zhang, Y., Cheng, C., Cheng, A.W., Zhang, X., Li, N., Xia, C., Wei, X., Liu, X., and Wang, H. (2017). CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. Cell Res 27, 154–157.
Ma, X., and Liu, Y.G. (2016). CRISPR/Cas9-based multiplex genome editing in monocot and dicot plants. Curr Protoc Mol Biol 115, 31.6.1–31.6.21.
Ma, X., Zhu, Q., Chen, Y., and Liu, Y.G. (2016). CRISPR/Cas9 platforms for genome editing in plants: developments and applications. Mol Plant 9, 961–974.
Ma, X., Chen, L., Zhu, Q., Chen, Y., and Liu, Y.G. (2015a). Rapid decoding of sequence-specific nuclease-induced heterozygous and biallelic mutations by direct sequencing of PCR products. Mol Plant 8, 1285–1287.
Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., Xie, Y., Shen, R., Chen, S., Wang, Z., Chen, Y., Guo, J., Chen, L., Zhao, X., Dong, Z., and Liu, Y.G. (2015b). A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant 8, 1274–1284.
Mao, H., Sun, S., Yao, J., Wang, C., Yu, S., Xu, C., Li, X., and Zhang, Q. (2010). Linking differential domain functions of the GS3 protein to natural variation of grain size in rice. Proc Natl Acad Sci USA 107, 19579–19584.
Mitsuda, S.H., and Shimizu, N. (2016). Epigenetic repeat-induced gene silencing in the chromosomal and extrachromosomal contexts in human cells. PLoS One 11, e0161288.
Miura, K., Ikeda, M., Matsubara, A., Song, X.J., Ito, M., Asano, K., Matsuoka, M., Kitano, H., and Ashikari, M. (2010). OsSPL14 promotes panicle branching and higher grain productivity in rice. Nat Genet 42, 545–549.
Monna, L., Kitazawa, N., Yoshino, R., Suzuki, J., Masuda, H., Maehara, Y., Tanji, M., Sato, M., Nasu, S., and Minobe, Y. (2002). Positional cloning of rice semidwarfing gene, *sd-1*: rice "green revolution gene" encodes a mutant enzyme involved in gibberellin synthesis. DNA Res 9, 11–17.
Patron, N.J. (2014). DNA assembly for plant biology: techniques and tools. Curr Opin Plant Biol 19, 14–19.
Piao, R., Jiang, W., Ham, T.H., Choi, M.S., Qiao, Y., Chu, S.H., Park, J.H., Woo, M.O., Jin, Z., An, G., Lee, J., and Koh, H.J. (2009). Map-based cloning of the *ERECT PANICLE 3* gene in rice. Theor Appl Genet 119, 1497–1506.
Qi, W., Zhu, T., Tian, Z., Li, C., Zhang, W., and Song, R. (2016). High-efficiency CRISPR/Cas9 multiplex gene editing using the glycine tRNA-processing system-based strategy in maize. BMC Biotechnol 16, 58.
Sakuma, T., Masaki, K., Abe-Chayama, H., Mochida, K., Yamamoto, T., and Chayama, K. (2016). Highly multiplexed CRISPR-Cas9-nuclease and Cas9-nickase vectors for inactivation of hepatitis B virus. Genes Cells 21, 1253–1262.
Shan, Q., Wang, Y., Li, J., and Gao, C. (2014). Genome editing in rice and wheat using the CRISPR/Cas system. Nat Protoc 9, 2395–2410.
Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J.L., and Gao, C. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31, 686–688.
Shen, L., Wang, C., Fu, Y., Wang, J., Liu, Q., Zhang, X., Yan, C., Qian, Q., and Wang, K. (2016). QTL editing confers opposing yield performance in different rice varieties. J Integr Plant Biol in press, doi: 10.1111/jipb.12501.
Si, L., Chen, J., Huang, X., Gong, H., Luo, J., Hou, Q., Zhou, T., Lu, T., Zhu, J., Shangguan, Y., Chen, E., Gong, C., Zhao, Q., Jing, Y., Zhao, Y., Li, Y., Cui, L., Fan, D., Lu, Y., Weng, Q., Wang, Y., Zhan, Q., Liu, K., Wei, X., An, K., An, G., and Han, B. (2016). OsSPL13 controls grain size in cultivated rice. Nat Genet 48, 447–456.
Song, X.J., Huang, W., Shi, M., Zhu, M.Z., and Lin, H.X. (2007). A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nat Genet 39, 623–630.
Sood, B.C., and Sidiq, E.A. (1978). A rapid technique for scent determination in rice. Indian J Genet Plant Breed 38, 268–275.
Spielmeyer, W., Ellis, M.H., and Chandler, P.M. (2002). Semidwarf (*sd-1*), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. Proc Natl Acad Sci USA 99, 9043–9048.
Wang, C., Shen, L., Fu, Y., Yan, C., and Wang, K. (2015a). A Simple CRISPR/Cas9 system for multiplex genome editing in rice. J Genets Genomics 42, 703–706.
Wang, S., Li, S., Liu, Q., Wu, K., Zhang, J., Wang, S., Wang, Y., Chen, X., Zhang, Y., Gao, C., Wang, F., Huang, H., and Fu, X. (2015b). The OsSPL16-GW7 regulatory module determines grain shape and simultaneously improves rice yield and grain quality. Nat Genet 47, 949–954.
Wang, X., Niu, Y., Zhou, J., Yu, H., Kou, Q., Lei, A., Zhao, X., Yan, H., Cai, B., Shen, Q., Zhou, S., Zhu, H., Zhou, G., Niu, W., Hua, J., Jiang, Y., Huang, X., Ma, B., and Chen, Y. (2016). Multiplex gene editing via CRISPR/Cas9 exhibits desirable muscle hypertrophy without detectable off-target effects in sheep. Sci Rep 6, 32271.
Wood, A.J., Lo, T.W., Zeitler, B., Pickle, C.S., Ralston, E.J., Lee, A.H., Amora, R., Miller, J.C., Leung, E., Meng, X., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D., and Meyer, B.J. (2011). Targeted genome editing across species using ZFNs and TALENs. Science 333, 307–307.
Wu, X., Tang, D., Li, M., Wang, K., and Cheng, Z. (2013). Loose plant architecture1, an INDETERMINATE DOMAIN protein involved in shoot gravitropism, regulates plant architecture in rice. Plant Physiol 161, 317–329.
Xing, H.L., Dong, L., Wang, Z.P., Zhang, H.Y., Han, C.Y., Liu, B., Wang, X.C., and Chen, Q.J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14, 327.
Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. (2000). *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the arabidopsis flowering time gene *CONSTANS*. Plant Cell 12, 2473–2484.
Yu, H., Murchie, E.H., González-Carranza, Z.H., Pyke, K.A., and Roberts,

J.A. (2015). Decreased photosynthesis in the *erect panicle 3* (*ep3*) mutant of rice is associated with reduced stomatal conductance and attenuated guard cell development. J Exp Bot 66, 1543–1552.
Zhang, Z., Mao, Y., Ha, S., Liu, W., Botella, J.R., and Zhu, J.K. (2016). A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in *Arabidopsis*. Plant Cell Rep 35, 1519–1533.
Zhou, Y., Zhu, J., Li, Z., Yi, C., Liu, J., Zhang, H., Tang, S., Gu, M., and Liang, G. (2009). Deletion in a quantitative trait gene *qPE9-1* associated with panicle erectness improves plant architecture during rice domestication. Genets 183, 315–324.

SUPPORTING INFORMATION

Figure S1 Mutation types at the eight target sites in the T_0 generation.

Table S1 Mutations detected in putative CRISPR/Cas9 off-target sites

Table S2 Primers used in this study.

The supporting information is available online at life.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.