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Letter to the Editor

Construction of a Genome-Wide Mutant Library in Rice Using CRISPR/Cas9

Dear Editor,

Rice (*Oryza sativa* L.) is one of the world's most important staple crops and a powerful model system for studying monocot species because of its relatively small genome, rich genomic resources, and a highly efficient transformation system. With the completion of rice genome sequencing, the challenge of the post-genomic era is to systematically analyze the functions of all rice genes. Gene knockout is a frequently used and effective strategy for achieving this goal. Thus, generation of large-scale mutants at the whole-genome level is of great value for both functional genomics and genetic improvement of rice. Traditionally, large numbers of mutants are produced by physical, chemical, or biological mutagenesis. Mutants created by these methods have made enormous contributions to basic plant research and crop improvement. T-DNA insertion (Jeon et al., 2000), TILLING (targeting-induced local lesions in genomes) (Till et al., 2007) and RNAi (RNA interference) (Wang et al., 2013) are the three most common methods of performing genetic studies. T-DNA insertion and TILLING are time-consuming and labor-intensive in generating genome-wide mutant libraries, because large mutagenized populations must be generated to ensure sufficient genome-wide coverage. In addition, the T-DNA insertions occur randomly and often in intergenic and noncoding regions, for TILLING mutants it is difficult to identify the targeted mutations for the observed phenotypes, and the RNAi method only reduces the expression of targeted genes rather than generating the knockout mutants. Recently, a simple and highly efficient genomic engineering tool, the CRISPR (Clustered Regularly Interspaced Palindromic Repeats)/Cas9 system, has been developed; this technology can create small insertions and deletions (indels) in specific target genes and has been applied to many organisms. Because it is an easy and convenient technique, some CRISPR/Cas9 mutant libraries have been developed for genome-wide mutation screens in cultured eukaryotic cells (Shalem et al., 2015). However, no large-scale CRISPR/Cas9 mutant libraries have yet been generated in higher plants. Here, we report the construction of a high-throughput CRISPR/Cas9 mutant library in rice and demonstrate its application for identifying gene functions and its potential use for genetic improvement.

Effective single-guide RNAs (sgRNA) targeting specific genomic sites using the CRISPR/Cas9 system are usually 20 bp in length and are followed by a protospacer adjacent motif (PAM) in the target with the sequence NGG. For rational design of highly specific sgRNAs, the 12-bp seed sequence of the sgRNA should match only one site in the target genome, so that there is only a low possibility of off-target editing (Doench et al., 2014). Using this criterion, we searched the rice genome and identified 1 535 852 target sites located in the exon regions of 52 916 rice genes. The CRIPSR/Cas9 system induces double-strand breaks and generally produces small indels, which often cause frameshifts in protein-coding sequences. Hence, to generate loss-of-function mutations efficiently, sgRNA target sites were designed in exons near the beginning of ORFs just downstream of start codons. We chose the first two identified sgRNA target sites in each candidate gene and selected 12 802 genes highly expressed in rice shoot base tissue (rice expression profiles database [RED]) and 25 604 corresponding sgRNAs to generate a large-scale mutant library.

We added 25-bp sequences (pVKmp-lib-FP0 and pVKmp-lib-RP0, Supplemental Table 1) derived from the plant expression vector pVKmp-lib (Supplemental Figures 1 and 2; ViewSolid Biotech) to two ends of the 20-bp guide sequences, respectively, for downstream PCR amplification and Gibson ligation, and synthesized 25 604 oligonucleotides of 70 bp by array-based oligonucleotide pool synthesis. To clone the synthesized oligo pool into the CRISPR/Cas9 binary vector pVKmp-lib, which carries the *hygromycin B phosphotransferase* (*hpt*) gene and the Cas9 expression cassette, the oligonucleotides were amplified by PCR using primer pVKmp-lib-FP1 and pVKmp-lib-RP1 (Supplemental Table 1). The purified PCR products were ligated into the pVKmp-lib vector by Gibson ligation (Figure 1A), and the plasmids were transformed into bacterial-host-competent cells and selected with kanamycin. More than 1.2×10^6 clones growing on the selection plates were harvested and combined to prepare the plasmid DNAs constituting the sgRNA library.

To evaluate the accuracy of the large-scale synthesized sgRNA, we randomly selected 275 clones from a total of 1.2×10^6 clones and sequenced them. The sequence data demonstrated that 20 of the 275 clones mutated sgRNA target sequences (7.3%), 16 clones multiplied sgRNA target sequences (5.8%) and one clone did not harbor in target sequence (0.4%) (Supplemental Table 2); the accuracy of the synthesized sgRNA was therefore 86.5%. Next, the coverage of the sgRNA library was assessed. The 303-bp PCR products generated from the plasmid DNA library with primers pVKmp-F1 and pVKmp-R1 (Supplemental Table 1) were deep sequenced by high-throughput sequencing. The sequence data showed that 25 265 of the 25 604 sgRNAs were represented by at least one read (98.7%), and these 25 265 sgRNAs covered 12 786 genes (99.9%). The majority (82.1%) of the sgRNAs had 11–90 reads, pointing to very high coverage and evenness of the sgRNA library (Figure 1B). These findings indicate that the constructed sgRNAs library is of high quality and with good gene coverage and sgRNA accuracy.

Then the plasmid DNA library was transformed to *Agrobacterium tumefaciens* strain EHA105 for rice transformation. We tested the accuracy and coverage of the sgRNA library in *Agrobacterium* by randomly selecting 390 clones and sequenced them. The results

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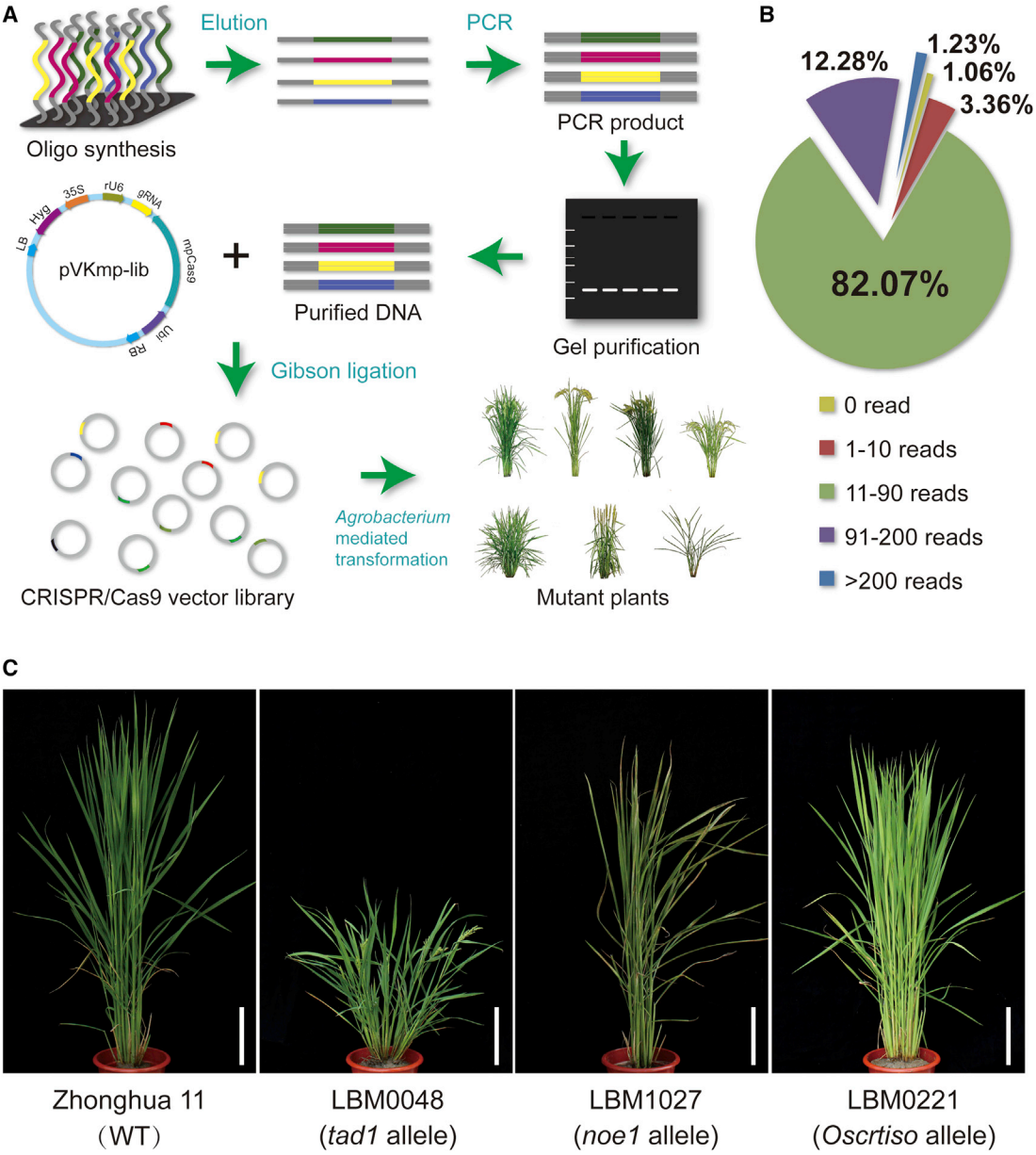

Figure 1. Construction of a Genome-Wide Mutant Library in Rice Using CRISPR/Cas9.
(A) Schematic illustration of the CRISPR/Cas9 rice library construction.
(B) Results of large-scale sequencing of the sgRNA library.
(C) Morphological phenotypes of the CRISPR/Cas9 rice mutants. The T_1 mutant plants showing abnormal phenotypes growing in normal field conditions in Beijing. Bar, 24 cm.

showed that 337 of the 390 clones harbored correct sgRNA target sequences (86.4%) (Supplemental Table 2), and from the 337 clones, 331 unique sgRNAs were detected, suggesting that the sgRNA library in *Agrobacterium* is of high accuracy and coverage. The rice variety Zhonghua 11 (*Oryza sativa* L. ssp. *japonica*) was selected as the recipient plant as it can be efficiently transformed and has good fertility, and its genome is fully sequenced. Embryonic calli that were derived from the scutella of mature seed embryos and divided vigorously during subculture were selected for *Agrobacterium*-mediated transformation. Transgenic seedlings generated from single infected calli were regarded as independent transgenic T_0 lines, and more than 14 000 independent T_0 lines were obtained. To evaluate the quality and coverage of the resulting mutant library, we selected 182 T_0 plants at random and sequenced their genotypes. We found that 139 T_0 plants harbored single correct sgRNAs, 38 plants harbored mutated sgRNAs, 4 plants harbored multiple sgRNAs, and one was non-transgenic (Supplemental Table 3). We also found that the 139 "correct" T_0 plants contained 136 different sgRNAs; thus only three sgRNAs were represented twice, demonstrating high coverage by the mutant library. We then focused on the knockout efficiency of the library using the DSDecode program (Liu et al., 2015); 46 plants were found to be homozygous mutant (33.1%),

15 plants were biallelic mutant (10.8%), and 27 were heterozygous mutant (19.4%) (Supplemental Figure 3 and Supplemental Table 4). Further analysis showed that most were small insertions or deletions at the target site; there were 65.3% (115/176) single-base insertions and deletions (Supplemental Figure 3), which is consistent with previous report (Ma et al., 2015). These results show that the CRISPR/Cas9 system is a powerful tool for constructing mutant libraries in rice.

In traditional mutant libraries, the relationship between genotype and phenotype is usually very weak, and it is difficult to identify and clone the genes corresponding to particular phenotypes. It is estimated that the tagging efficiency of a typical rice T-DNA insertion library may be as low as 5%–10% (Wei et al., 2016). Even though RNAi mutant libraries can have higher mutation rates (about 47.9% lines with observable phenotypes), mutations usually result from silencing of any one of several members of a gene family rather than of individual target genes (Wang et al., 2013). To evaluate the relationship between genotype and phenotype in the CRISPR/Cas9 mutant library, we harvested all the seeds from these 14 000 T_0 lines and randomly selected 200 lines for phenotyping by planting 24 plants per line in the field. Apparent morphological differences from the wild-type in terms of plant height, tilling, heading time, and leaf color were observed among the progeny of 54 T_0 plants. Sequencing revealed that 32 of the 54 plants harbored the correct target sgRNA vectors and had been edited by the CRISPR/Cas9 system (Supplemental Table 5). For example, 11 individual T_1 plants from the T_0 plant LBM0048 had increased tiller numbers, reduced heights, and twisted leaves (Figure 1C and Supplemental Table 6), and Sanger sequencing showed that these T_1 mutants were *tad1* homozygotes, and their phenotype was consistent with that of previously described mutants (Xu et al., 2012). Similarly, four T_1 plants from T_0 plant LBM1027 had damaged leaves with white variegated areas and cell death (Figure 1C and Supplemental Table 6). Further analysis revealed that these plants were *noe1* homozygotes, although their phenotype had previously only been seen when *noe1* mutants were cultivated under strong light (Lin et al., 2012). Also, six T_1 plants from the T_0 plant LBM0221 had altered green and yellow cross bands on the leaf blades (Figure 1C and Supplemental Table 6), and were identified as *OsCRTISO* homozygotes, in perfect agreement with a previous report (Fang et al., 2008).

These examples indicate that the genotype and phenotype are much more closely related in the present large-scale CRISPR/Cas9 knockout mutant rice library than in previous mutant resources. Because there is an almost one-to-one relationship between guide sequence and targeted gene among these CRISPR/Cas9 mutants, the CRISPR/Cas9 mutant library requires less laborious mapping to identify interesting genes. Moreover, independent mutant alleles of the same gene can be recognized by their similar phenotypes, so avoiding tedious complementation analysis.

Finally, we tested the off-target effects of the rice mutants, which is a main concern for the CRISPR/Cas9 system. We computationally predicted the genome-wide potential off-target sites for LBM0048, LBM0221, and LBM1027 using the Cas-OFFinder (Bae et al., 2014) tool in rice. Eight likely off-target sites with three nucleotide mismatches to the recognition site of LBM1027-sgRNA were identified; similarly, five and nine off-target sites were screened for LBM0221 and LBM0048-sgRNA, respectively (Supplemental Table 7). Using sequencing, the off-target effects were measured for T_0 mutants, and the results revealed that none of these sites was mutated among the LBM0048, LBM0221, and LBM1027 mutants (Supplemental Table 7). These data suggested that the CRISPR/Cas9 mutant library we obtained has the low off-target effects, which may be due to the careful sgRNA filtering and rational design in the beginning.

In summary, we have constructed a large-scale CRISPR/Cas9 mutant library in rice that is of high quality, with good coverage and uniform distribution. Our study demonstrates that CRISPR/Cas9-based screening is a robust method for systematically identifying both functional genes and mutant phenotypes in rice. The mutant library developed here will be of a great value for the study of gene functions in rice and for crop improvement.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

X.M., X.S., F.Z., and S.G. performed the experiments; Y.H. conducted the bioinformatics analyses; X.M., Y.H., Y.Z., C.G., and J.L. designed the experiments and wrote the paper.

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Xiangbing Meng[1,5], Hong Yu[1,5], Yi Zhang[2,4], Fengfeng Zhuang[3], Xiaoguang Song[1], Songsong Gao[3], Caixia Gao[2,] and Jiayang Li[1,4,*]*

[1]State Key Laboratory of Plant Genomics and National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[2]State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[3]Beijing ViewSolid Biotechnology, Beijing 102206, China
[4]University of the Chinese Academy of Sciences, Beijing 100049, China
[5]These authors contributed equally to this article.
***Correspondence: Caixia Gao (cxgao@genetics.ac.cn), Jiayang Li (jyli@genetics.ac.cn)**

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