CellPress
PARTNER JOURNAL

Molecular Plant
Research Article

A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants

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http://dx.doi.org/10.1016/j.molp.2015.04.007

ABSTRACT

CRISPR/Cas9 genome targeting systems have been applied to a variety of species. However, most CRISPR/Cas9 systems reported for plants can only modify one or a few target sites. Here, we report a robust CRISPR/Cas9 vector system, utilizing a plant codon optimized *Cas9* gene, for convenient and high-efficiency multiplex genome editing in monocot and dicot plants. We designed PCR-based procedures to rapidly generate multiple sgRNA expression cassettes, which can be assembled into the binary CRISPR/Cas9 vectors in one round of cloning by Golden Gate ligation or Gibson Assembly. With this system, we edited 46 target sites in rice with an average 85.4% rate of mutation, mostly in biallelic and homozygous status. We reasoned that about 16% of the homozygous mutations in rice were generated through the non-homologous end-joining mechanism followed by homologous recombination-based repair. We also obtained uniform biallelic, heterozygous, homozygous, and chimeric mutations in *Arabidopsis* T_1 plants. The targeted mutations in both rice and *Arabidopsis* were heritable. We provide examples of loss-of-function gene mutations in T_0 rice and T_1 *Arabidopsis* plants by simultaneous targeting of multiple (up to eight) members of a gene family, multiple genes in a biosynthetic pathway, or multiple sites in a single gene. This system has provided a versatile toolbox for studying functions of multiple genes and gene families in plants for basic research and genetic improvement.

Key words: sequence-specific nucleases, genome editing, CRISPR/Cas9, rice, *Arabidopsis*

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. (2015). A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. Mol. Plant. **8**, 1274–1284.

INTRODUCTION

Mutants are critical for genetic analysis to study gene function in both basic and applied research. Compared with the traditional inefficient and laborious random mutagenesis and screening, targeted gene editing technologies can significantly facilitate the process for creating mutants of target genes. Previous studies have shown that double-strand breaks (DSB) in the nuclear DNA can trigger two independent endogenous DNA repair pathways: non-homologous end joining (NHEJ) and homologous recombination (HR), which can frequently result in small or large chromosomal changes (Sonoda et al., 2006). When the chromosomal changes occur within genes, loss-of-function or weakened gene mutations can be created.

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and IPPE, SIBS, CAS.

In recent years various genetic tools, especially those involving sequence-specific nucleases for creating targeted DSB, have emerged as the major breakthrough for site-specific genome editing (Li et al., 2011; Wood et al., 2011; Cong et al., 2013). For example, the Zinc-finger nucleases (ZFNs) and transcription activator-like (TAL) effector nucleases (TALENs) combine the programmable DNA binding domains and the cleavage domain of the endonuclease *Fok*I to form chimeric proteins. ZFNs utilize triple-arrayed zinc-finger domains for DNA recognition, and each zinc-finger domain recognizes three base pairs. On the other hand, TALENs utilize TAL effectors adapted from plant pathogenic *Xanthomonas* for DNA binding. The DNA recognition units in TAL effectors are nearly identical tandem repeat units; each unit recognizes one nucleotide (Boch and Bonas, 2010). Successful gene targeting has been reported using ZFNs and TALENs in animals and plants (Li et al., 2012; Shan et al., 2013a; Wang et al., 2013a, 2013b, 2013c). However, constructing the binding domain of ZFNs is technically difficult (Strauß and Lahaye, 2013). On the other hand, the repetitive property of the binding domain in TALENs is complicated and also causes concerns regarding vector stability in bacterial and plant genomes. In addition, the fact that both ZFNs and TALENs function as dimers makes the processes of vector construction and transformation more complicated.

Recently, a newly established gene editing tool, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, derived from the adaptive immunity system of *Streptococcus pyogenes*, has provided a breakthrough (Jinek et al., 2012). The Cas9 protein functions as a nuclease and is directed to a target site by an engineered sequence-specific single guide RNA (sgRNA). This site-specific targeting is determined by the first 20 nucleotides (target seed sequence) of the sgRNA, rather than protein binding domains as with ZFNs and TALENs, for the DNA recognition (Jinek et al., 2012). CRISPR/Cas9 editing systems have enabled genomic targeting in many organisms, including plants (Cong et al., 2013; Jiang et al., 2013a, 2013b; Li et al., 2013; Mao et al., 2013; Miao et al., 2013; Shan et al., 2013b; Xie and Yang, 2013; Fauser et al., 2014; Zhang et al., 2014; Zhou et al., 2014). More importantly, multiple sgRNAs designed with different target sequences can direct Cas9 to specific corresponding sites (Cong et al., 2013; Wang et al., 2013a, 2013b, 2013c). This feature is especially important because the innate ability of Cas9 to edit multiple loci simultaneously in the same individual has many potential applications in both basic and applied research, such as mutation of multiple members of gene families or functionally related genes that control complex traits.

Different from genome editing in animals and microorganisms, CRISPR/Cas9-based genomic editing in plants usually depends on stable transformation with constructs expressing Cas9 and sgRNA(s), mostly using *Agrobacterium*-mediated transformation. Recently, a virus based vector system has been used for gene targeting in plants, which provides an alternative strategy for delivery of *Cas9* and sgRNA expression cassettes (Ali et al., 2015; Honig et al., 2015). Although the *Cas9* and sgRNA expression cassettes can be combined into a single T-DNA region, current plant CRISPR/Cas9 vector systems can only target one or few genomic sites (Jiang et al., 2013a, 2013b; Li et al., 2013; Mao et al., 2013; Shan et al., 2013a, 2013b; Xie and Yang, 2013; Fauser et al., 2014; Feng et al., 2014; Xing et al., 2014; Zhang et al., 2014; Zhou et al., 2014). Here, we report a plant CRISPR/Cas9 vector system that enables efficient editing of multiple genes in monocot and dicot plants. Using this system, we can efficiently assemble multiple sgRNA expression cassettes into a single binary CRISPR/Cas9 vector, in one round of cloning, by Golden Gate ligation (Engler et al., 2008) or Gibson Assembly (Gibson et al., 2009). We show that this system can uniformly, efficiently, and simultaneously produce multiple heritable mutations in T_0 rice and *Arabidopsis* T_1 plants by targeting multiple genes or genomic sites via single transformation events.

RESULTS

CRISPR/Cas9 Multi-Targeting Vectors for Monocot and Dicot Plants

In Gramineae genomes, most genes have higher GC contents in the 5′ terminal regions of their open reading frames (ORFs) (Wong et al., 2002). Mimicking this feature, we designed and synthesized a plant codon optimized *Cas9* gene (*Cas9p*) with higher GC contents (62.5%) in the 5′ region (400 bp) and 54.2% overall GC contents (Figure 1A and Supplemental Figure 1). Other *Cas9* genes used for plant genome targeting (Li et al., 2013; Mao et al., 2013; Shan et al., 2013a, 2013b; Xie and Yang 2013; Zhou et al., 2014) lack such a feature of the 5′ GC enrichment (Supplemental Figure 2). We used *Cas9p* to construct five binary CRISPR/Cas9 vectors, pYLCRISPR/Cas9P_{ubi}-H, pYLCRISPR/Cas9P_{ubi}-B, pYLCRISPR/Cas9P_{35S}-H, pYLCRISPR/Cas9P_{35S}-N, and pYLCRISPR/Cas9P_{35S}-B, in which *Cas9p* is driven by the maize ubiquitin promoter (P_{ubi}) or the cauliflower mosaic virus 35S promoter (P_{35S}). These vectors contain various plant-selectable marker genes and each has two *Bsa*I sites that flank a toxic *ccdB* gene (a negative selectable marker) for cloning of sgRNA expression cassette(s) (Figure 1B).

To facilitate the expression of multiple sgRNA cassettes in CRISPR/Cas9 constructs, we cloned the U3 and U6 small nuclear RNA promoters from rice: *OsU3*, *OsU6b*, *OsU6c* (Shan et al., 2013a, 2013b; Xie and Yang, 2013; Zhou et al., 2014), and *OsU6a* (identified in this study). We also cloned four *Arabidopsis* U3 and U6 promoters (Waibel and Filipowicz, 1990). Using these promoters, we prepared a set of six sgRNA intermediate vectors suitable for gene targeting in monocot plants, and another set of six sgRNA vectors for genome targeting in dicot plants (Figure 1C and 1D). The *LacZ* marker gene in four of the sgRNA vectors can be used to facilitate selection of *Escherichia coli* positive CRISPR/Cas9 clones carrying the sgRNA expression cassettes.

The sgRNAs with target sequences (target-sgRNAs) are transcribed from the U3 or U6 promoters with definite transcription initiation sites, e.g., A nucleotide for U3 promoters and G nucleotide for U6 promoters (Cong et al., 2013; Shan et al., 2013a, 2013b). Therefore, target sequences are commonly selected in the genomes with 5′-AN(19)NGG (NGG: protospacer adjacent motif, PAM) for the U3 promoters and 5′-GN(19)NGG for the U6 promoters. It was reported that target-sgRNAs with extended nucleotides at the 5′ end (derived from the vector ligation site) could also guide genome editing in plants (Xie and Yang, 2013), but it is

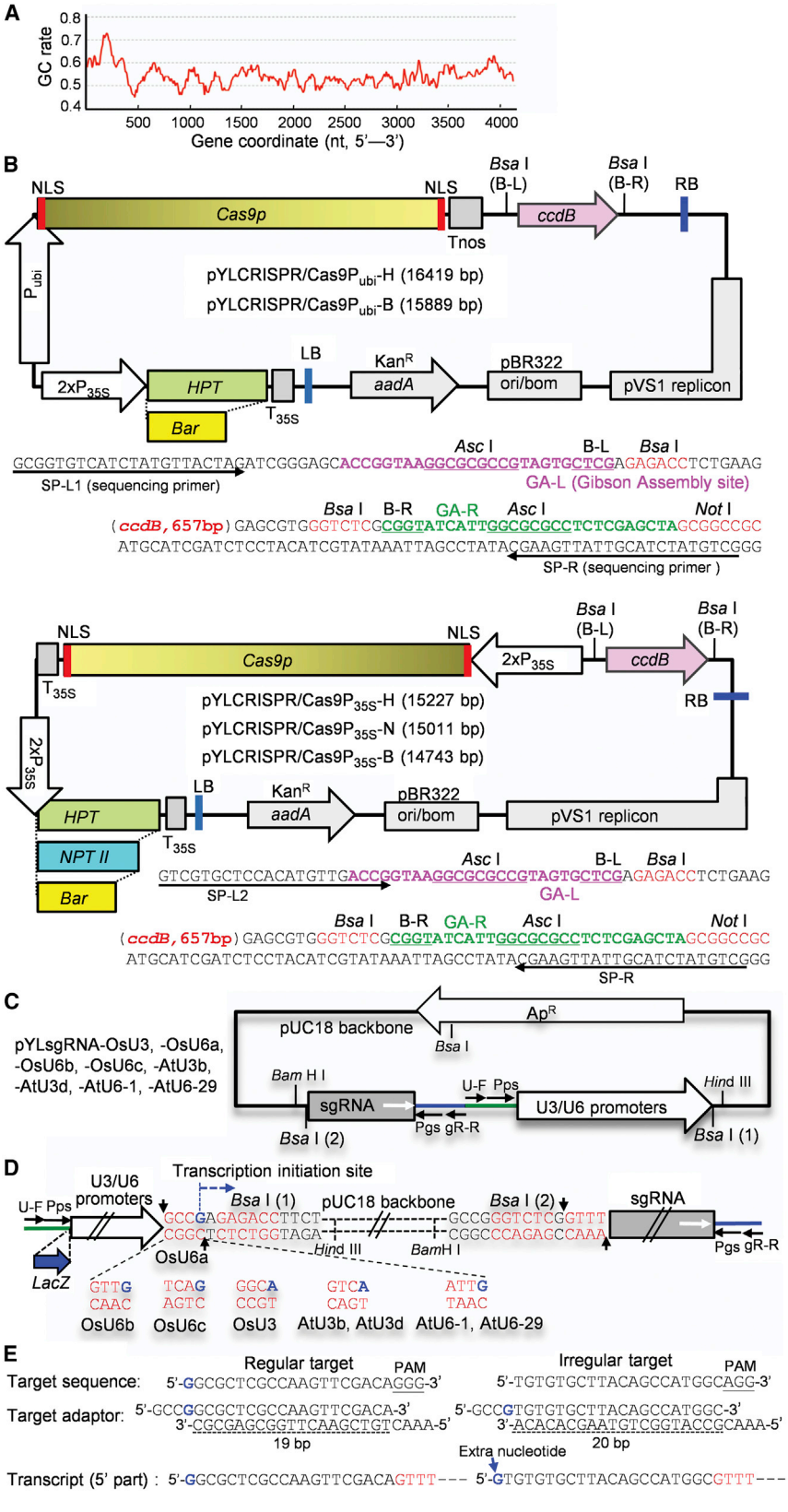

Figure 1. A CRISPR/Cas9 System for Monocot and Dicot Plants.

(A) Distribution of GC content in *Cas9p* scanned by 100-nt sliding windows with 10-nt overlap between windows.

(B) Structures of the pYLCRISPR/Cas9 binary vectors based on the pCAMBIA1300 backbone. *HPT* (-H), *Bar* (-B), and *NPT II* (-N) encode hygromycin B phosphotransferase, PPT acetyltransferase, and neomycin phosphotransferase II, respectively. NLS, nuclear localization sequence; adapted from Cong et al. (2013). The key sequences and restriction sites for cloning and analysis of sgRNA expression cassettes are given.

(C) Overall structure of the sgRNA intermediate vectors.

(D) The *Bsa*I-cutting sequences are given in 12 sgRNA vectors (in a linear form), including four other vectors (pYLsgRNA-OsU3/LacZ, pYLsgRNA-OsU6a/LacZ, pYLsgRNA-AtU3b/LacZ, and pYLsgRNA-AtU3d/LacZ) that have an additional *LacZ* gene (198 bp) as a cloning selection marker. The U3 and U6 promoters from rice (Os) and *Arabidopsis* (At) and the sgRNA sequence are separated by the vector backbone, to avoid amplification of the uncut plasmids by PCR with a short extension time during preparation of the sgRNA expression cassettes. Cutting (small arrows) of the plasmids with *Bsa*I produces distinct non-palindromic sticky ends to the promoters and a common end to the sgRNA sequence.

(E) A representative regular target and an irregular target, their target adaptors for the OsU6a promoter, and the transcribed 5′ sequences are shown. A ligated target-sgRNA expression cassette is amplified by nested PCR using primers U-F/gR-R and Pps/Pgs. Pps and Pgs are position-specific primers with distinct *Bsa*I-cutting sites for the Golden Gate ligation (Supplemental Table 2), or with different overlapping ends for Gibson Assembly (Supplemental Table 3).

unclear whether this kind of target-sgRNAs affect the editing efficiency. Therefore, we also selected some targets with a starting nucleotide T or C, termed irregular targets here, in the genome to test the editing efficiency. Transgenic plants with these irregular target-containing constructs transcribe target-sgRNAs carrying an extra A or G nucleotide at the 5′ end [5′-(A/G)(C/T)N(19)-sgRNAs], compared with the regular ones [5′-(A/G)N(19)-sgRNAs] (Figure 1E).

Preparation of CRISPR/Cas9 Constructs for Multiplex Editing in Rice and *Arabidopsis*

To generate sgRNA expression cassettes containing the target sequences, the sgRNA vectors with the selected U3 and/or U6 promoters were digested with *Bsa*I and ligated with double-stranded target adaptors (Figure 1D and 1E). Alternatively, target sequences were introduced into sgRNA expression cassettes by overlapping PCR with target sequence-containing chimeric primers (Supplemental Figure 4 and Supplemental Table 1). The integrated sgRNA expression cassettes were amplified by nested PCRs; the secondary PCR

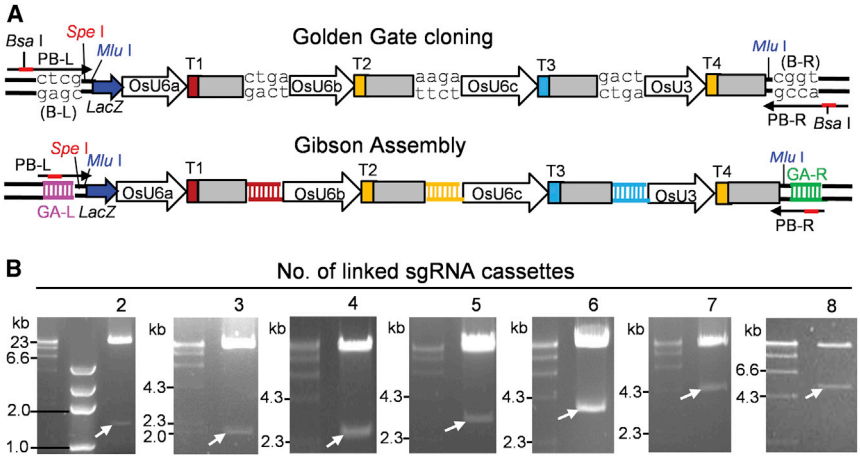

Figure 2. Cloning of sgRNA Expression Cassettes into the CRISPR/Cas9 Binary Vectors.
(A) Illustration of cloning of four sgRNA expression cassettes into the CRISPR/Cas9 binary vectors by single Golden Gate ligation or Gibson Assembly. The U3/U6 promoters can be ordered arbitrarily. The *Spe*I site is unique in the construct introduced by the first Pps primer (Supplemental Tables 1 and 2), and can be used for insertion of additional sgRNA expression cassettes, if necessary, by Gibson Assembly. The ligated products are directly transferred into *E. coli* competent cells. Alternatively, the ligated products of multiple sgRNA expression cassettes with the binary vector can be firstly amplified using primers PB-L and PB-R, and then again digested and ligated to the vector, to increase the cloning efficiency.
(B) Agarose gel analysis of CRISPR/Cas9 constructs with different numbers of linked sgRNA expression cassettes (arrows), which were digested with *Mlu*I or *Asc*I.

used site-specific primers (Pps, Pgs), which include primers with different *Bsa*I-cutting sites (Supplemental Table 2) for Golden Gate ligation (Engler et al., 2008). Golden Gate ligation uses the special cleavage feature of type IIs restriction endonucleases, such as *Bsa*I, to design and generate distinct, non-palindromic sticky ends of sequences, which can avoid self-ligation and non-compatible end ligation. Thus, this method is efficient for linking multiple DNA fragments in a designed order in a single reaction (Figure 2A). Another set of site-specific primers (Supplemental Table 3) was also designed for isothermal *in vitro* recombination, also called Gibson Assembly (Gibson et al., 2009), which has the powerful capability to assemble multiple DNA fragments with overlapping ends (Figure 2A).

Using these cloning strategies, we prepared 20 pYLCRISPR/Cas9P_{ubi}-H-based constructs, each carrying one or multiple (up to eight) sgRNA expression cassettes driven by the OsU3 and OsU6 promoters for gene targeting in rice (Figure 2B and Supplemental Table 3). In addition, four pYLCRISPR/Cas9P_{35S}-H-based constructs carrying one to three sgRNA expression cassettes driven by the AtU3 and/or AtU6 promoters were also prepared for targeting genes in *Arabidopsis* (Supplemental Table 4).

We transferred these 20 constructs into rice cultivars by *Agrobacterium*-mediated transformation (Hiei et al., 1994). To test whether this system can also effectively edit genomic sites in dicots, we transformed *Arabidopsis thaliana* with the four constructs using the *Agrobacterium*-infiltration (floral dip) method (Clough and Bent, 1998).

Characterization of Targeted Editing in Transgenic Rice and *Arabidopsis* Plants

We analyzed rice T_0 (the first transgenic generation) plants from these constructs involving in 46 genomic targets, by direct sequencing of PCR products containing the targeted sites. Heterozygous (wild-type/single mutation) and biallelic (two distinct variants) mutations that produced superimposed sequencing chromatograms (Supplemental Figure 5) were identified using our Degenerate Sequence Decoding method (Ma et al., 2015), with some samples further verified by sequencing individual amplicon-containing plasmid clones. In 328 sequenced sites, 280 (85.4%) had uniform mutations, which included: 177 (54.9%) biallelic, 81 (24.7%) homozygous, and 19 (5.8%) heterozygous mutations (Figure 3A and Supplemental Tables 4 and 5). Only one T_0 plant had chimeric mutations, in which at least three distinct alleles of the targeted site were detected.

We tried to find the parameters that affect the Cas9 targeting efficiency from our data set. First, we observed that the sites targeted by the different promoter-driven sgRNAs had similar mutation rates of 81.4%–90.0% (Figure 3B), indicating that all these promoters, including *OsU6a* newly identified in this study, are effective in driving the genome editing. Second, since CRISPR/Cas9-based genomic editing relied on the binding of a sgRNA to its target site, we analyzed the editing efficiency of targets with different GC contents. The targets with GC contents higher than 50% had relatively higher editing efficiencies (88.5%–89.6%) than the editing efficiency (77.2%) of targets with GC contents lower than 50% (Figure 3B). Third, we compared the relative editing efficiencies between regular targets (i.e., those with a G [U6 promoter] or A [U3 promoter] as the starting nucleotide) and irregular ones (i.e., those having a T or C as the starting nucleotide), and found that they had similar editing efficiencies (84.3% and 86.9%, respectively) (Figure 3C and Supplemental Table 4). This finding broadens the selection of targets for efficient genomic editing, which are not restricted to the regular targets of 5′-AN(19)NGG for the U3 promoters and 5′-GN(19)NGG for the U6 promoters.

We noted that three target sites for *OsFTL11*, *Os07g0261200*, and *Os02g0700600* showed no editing or extremely low rates of editing in the rice T_0 plants. The failed editing of *OsFTL11* was probably due to a much lower sgRNA level (see below). The lower editing efficiency of *Os07g0261200* might be because of the low GC content (35%) in the target sequence. It is proposed that the secondary structures of sgRNAs are critical for

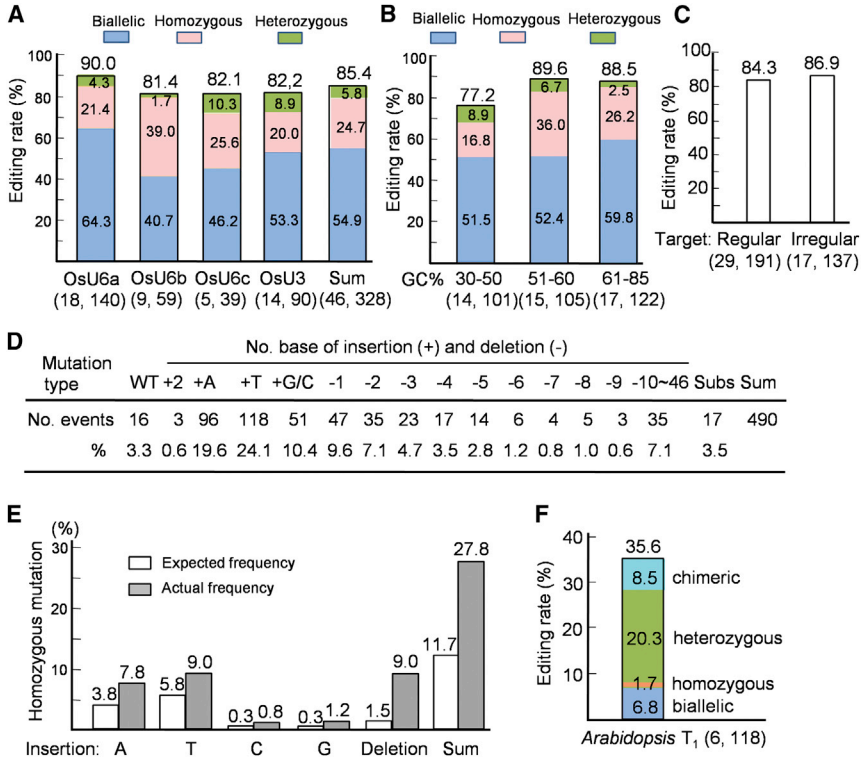

Figure 3. Characterization of Targeted Editing in Rice and *Arabidopsis*.

(A) Editing efficiencies of different allele types driven by different U3/U6 promoters in rice T_0 plants. Figures in parentheses (including those in **B**, **C**, and **F**) are the numbers of involved targets and sequenced site, respectively.

(B) Editing efficiencies of targets with different GC contents.

(C) Editing efficiencies of the regular and irregular targets.

(D) Frequencies of different editing events, which were calculated from 245 mutated sites (not including those of homozygous wild-type and fragmental deletion between two targeted sites). Subs, nucleotide substitution.

(E) Expected and actual frequencies of homozygous mutations. For the expected frequencies of homozygous mutations, calculated by square of the frequency of the each mutation type shown in **(D)**, it was assumed that two identical mutated alleles were produced independently at both homologous chromosomal sites by the NHEJ mechanism. The expected frequencies of the homozygous mutations with deletion of three and more bases were very rare, and thus were omitted from the calculation.

(F) Editing efficiencies of different mutation types in *Arabidopsis* T_1 plants.

Cas9/sgRNA effectiveness (Makarova et al., 2011). Therefore, we analyzed the secondary structures of all target-sgRNAs. Most high-efficiency targets had no pairing to the sgRNA sequence or pairing with less than continuous 6 bp (Supplemental Table 4). However, the target-sgRNA for *Os02g0700600* formed a typical stem-loop structure with pairing of continuous 14 and 4 bp of the target to the sgRNA sequence (Supplemental Figure 6). This stem-loop structure might inhibit the binding of the sgRNA to the target strand, thus leading to failure of the editing. Therefore, selection of target sequences should avoid those with pairing to the sgRNA by more than continuous 6 bp.

We observed a variety of types of targeted mutations, including insertion of one or two nucleotides and deletion of different numbers of nucleotides (Figure 3D and Supplemental Table 5). In 245 analyzed mutated sites (490 alleles in total, not including those of homozygous wild-type and fragmental deletion between two close targeted sites), a high proportion (54.1%) of the mutation events were single-base insertions, of which most inserted an A or T nucleotide (Figure 3D), consistent with a previous report (Zhang et al., 2014). In 20 cases with two or more target sites in single genes, the fragments between the target sites were deleted (Supplemental Figure 5 and Supplemental Table 5).

Our T_0 rice mutation library had a high frequency of homozygous mutations, 24.7% of the total sites (Figure 3A) or 27.8% of the 245 mutated sites (Figure 3E). To investigate whether such high proportion of homozygous mutations resulted from independent mutations at both homologous chromosomal sites by the NHEJ repair pathway, we calculated and compared the expected and actual frequencies of the homozygous mutations. The results showed that the total expected homozygous mutation frequency was 11.7% (Figure 3E), much lower than the actual frequency (27.8%), suggesting that HR-based repair mechanism might also involve the production of the homozygous mutations.

We analyzed the targeted editing in *Arabidopsis* T_1 (the first transgenic generation) plants derived from the four constructs for targeting six sites in four genes (Supplemental Table 4). In 118 sequenced sites, 42 (35.6%) detected mutations, with 8 (6.8%) biallelic, 2 (1.7%) homozygous, 24 (20.3%) heterozygous, and 10 (8.5%) chimeric mutations (Figure 3F, Supplemental Figure 7, and Supplemental Table 5). Similar to the target site in *Os07g0261200* with low GC content mentioned above, one of the three target sites in an *Arabidopsis* gene (*At5g55580*) with 25% of GC also had a lower editing rate (2/9).

The Edited Alleles in Rice and *Arabidopsis* Are Heritable

We analyzed three T_1 lines involving eight targeted sites, the results of which showed stable inheritance of the edited sites, which fitted the expected segregation ratio except for one site that was heterozygous in the T_0 generation (Supplemental Table 6). In this T_1 line, the frequency of the wild-type allele of this site was less than expected, while two new mutated alleles were detected (Supplemental Table 6), indicating that the wild-type site was further edited in low efficiency during growth of the transgenic plant.

Analysis of the targeted site of an *Arabidopsis* gene (*At1g16210*) in four T_2 lines showed that the edited alleles were inherited in the progeny, though showing allele frequencies lower than those expected (Supplemental Table 7). Various new editing events were

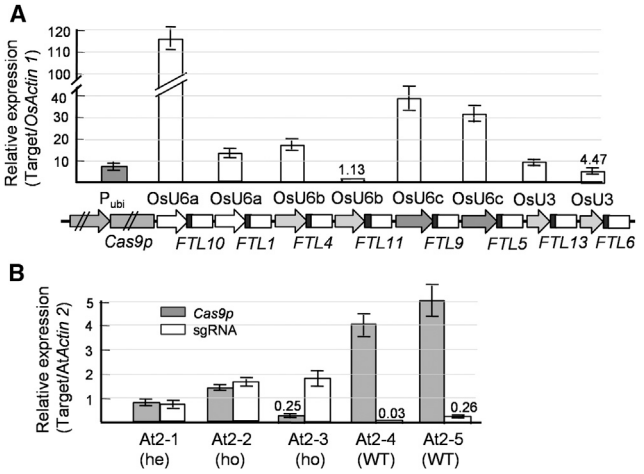

Figure 4. RT–qPCR Measurement of Expression of *Cas9p* and sgRNA.

(A) *Cas9p* and sgRNAs were driven by the P_{ubi} and OsU3/U6 promoters, respectively, in a callus from a T_1 seed of Os1-1 transformed with the eight-target construct for targeting the *OsFTL* genes. Except for *OsFTL11*, all of the genes had mutations (Supplemental Table 5). *OsActin1* was used as the internal control.

(B) *Cas9p* and sgRNA were driven by the P_{35S} and AtU6-29 promoters, respectively, in leaves of five *Arabidopsis* T_1 plants targeting *At1g56650*, which had heterozygous mutation (he), homozygous mutation (ho), or wild-type (WT) of the targeted site (Supplemental Table 5). *AtActin2* was used as the internal control.

detected in these lines that carried the wild-type allele of the heterozygous mutations.

Expression Analysis of *Cas9p* and sgRNAs

We used reverse transcription–quantitative PCR (RT–qPCR) to measure the expression of *Cas9p* and sgRNAs in rice callus with an eight-target construct targeting the *FTL* genes (see below). The expression level of *Cas9p* driven by the P_{ubi} promoter was about seven-fold that of the high-level expression gene *OsActin1* (Figure 4A). The OsU3 and OsU6 promoters showed high expression levels of the sgRNAs, mostly more than four-fold that of *OsActin 1*. The similar editing efficiencies among sgRNAs driven by these promoters (Figure 3A) suggest that the abundance of the sgRNAs is not a limiting factor in most cases, with the exception of the *OsFTL11*-targeting sgRNA that had much lower level of expression (Figure 4A). We also analyzed the *Cas9p* and sgRNA expressions in five *Arabidopsis* T_1 plants for targeting the same site of a gene (*At1g56650*). The expression levels of *Cas9p* driven by the P_{35S} promoter were 0.25–5.05-fold that of *AtActin2*, and the sgRNA levels driven by AtU6-29 ranged from 0.26- to 1.75-fold that of *AtActin2* (Figure 4B). It is notable that two plants (At2-4, At2-5) that failed in the editing had very low levels of the sgRNA, and an *Arabidopsis* T_1 plant (At2-3) had a low level (0.25-fold) of the *Cas9p* mRNA yet a relatively high sgRNA level (1.75-fold that of *AtActin2*) (Figure 4B), but this plant produced a homozygous mutation (Supplemental Figure 7). These results suggested that the expression level of this target-sgRNA, but not *Cas9p*, might be the limiting factor for genome editing in the *Arabidopsis* T_1 plants.

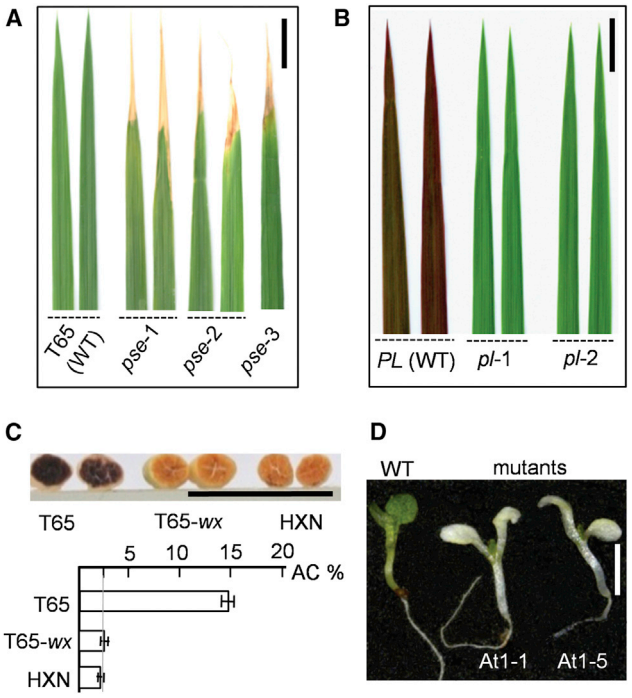

Figure 5. Phenotypic Variations of Mutants Generated from the CRISPR/Cas9 Targeting.

(A) Flag leaves at heading stage of three T_0 *premature senescence* (*pse-1*∼ *pse-3*) mutants and the parental line T65 (*japonica*). The mutants had loss-of-function mutations of seven *FTL* genes (except for *OsFTL11*). Scale bar, 1 cm.

(B) Knockout of *OsGSTU*, *OsMRP15*, and *OsAnP* in a purple leaf rice line (PL) destroyed the anthocyanin synthesis pathway in the mutants (*pl-1*, *pl-2*). Scale bar, 1 cm.

(C) Cut seeds of T65, an *OsWaxy*-knockout mutant (T65-*wx*), and a natural glutinous rice variety HXN were stained with 1% I_2-KI solution. Amylose contents (AC) of seeds of the lines were measured.

(D) Albino phenotype of two *Arabidopsis* T_1 mutants with biallelic mutations at the first site [At1-1(1)] or the first and second sites [At1-5(1), At1-5(2)] of the three target sites within the coding region of *At5g55580* (Supplemental Table 5). Scale bar, 2 mm.

Generation of Phenotypic Mutants in Rice and *Arabidopsis* by Targeting Multiple Genes or Multiple Sites of One Gene

Using our targeted editing system, we created mutants involving three sets of rice genes and one gene in *Arabidopsis*. First, rice has an *FT-like* (*FTL*) gene family with 13 members, but their functions remain unclear, except for *Hd3a* and *RFT1* that encode the homologs of *Arabidopsis* FT as the florigens for flowering (Tamaki et al., 2007). To study their functions, we prepared two constructs with eight and three sgRNA expression cassettes to target these 11 *FTL* genes (Supplemental Table 4). Sequencing analysis showed that all genes except *OsFTL11*, had mutations in the T_0 plants (Supplemental Tables 4 and 5). In all of the three obtained T_0 plants with the eight-target construct, seven genes (*OsFTL1*, *OsFTL4*, *OsFTL5*, *OsFTL6*, *OsFTL9*, *OsFTL10*, and *OsFTL13*) had frame-shift mutations in their ORFs (Supplemental Table 5), and these mutants showed a phenotype of premature leaf senescence (Figure 5A). These mutants provide valuable material for further study of the functions of rice *FTL* genes.

Second, we examined rice genes involved in anthocyanin accumulation. *OsGSTU* and *OsMRP15* are homologs of maize *Bronze 2* and *ZmMRP3*, encoding a glutathione *S*-transferase involved in anthocyanin accumulation (Marrs et al., 1995) and an anthocyanin transporter (Zhu et al., 2013), respectively. *OsAnP* is a homolog of *Arabidopsis AtTT12* and encodes a transporter mediating vacuolar sequestration of proanthocyanins (Debeaujon et al., 2001). When we mutated these genes in a rice line with purple leaves (Supplemental Tables 4 and 5) the mutants had green leaves (Figure 5B), confirming the role of these genes in anthocyanin synthesis in rice.

Third, we destroyed the *OsWaxy* gene, which functions in the synthesis of amylase (Wang et al., 1990), by transforming a *japonica* rice cultivar Taichung 65 (T65) with a construct targeting three sites in *OsWaxy*. Analysis of seeds (T_1 generation) of T_0 plants that had mutations in one or two of the target sites within the *OsWaxy* gene (Supplemental Tables 4 and 5) showed that the amylose content decreased from 14.6% in T65 to 2.6% in the mutants, similar to a natural glutinous rice variety (Figure 5C).

Finally, we targeted an *Arabidopsis* new gene (*At5g55580*), which encodes a putative mTERF (mitochondrial transcription termination factor) protein of unknown function, with three targets set on the exons. Two resulting small T_1 seedlings showed an albino phenotype (Figure 5D). Further sequencing analysis detected biallelic mutations at one (At1-1) or two (At1-5) of the three targets (Supplemental Figure 7 and Supplemental Table 5), indicating that this gene is essential for chloroplast biogenesis.

DISCUSSION

We have developed a comprehensive, robust, and easy-to-use CRISPR/Cas9 system for targeting multiple genes and genomic sites in monocots and dicots. Five binary vectors with the *Cas9p* gene driven by P_{ubi} or P_{35S} promoters coupled with different plant-selectable marker genes, and different sgRNA promoters, are available for the targeting of various plant species. The backbone of our binary CRISPR/Cas9 vectors is based on the widely used pCAMBIA1300 vector, which has the capacity of maintaining relatively large inserts (up to about 20 kb) in *E. coli* and *Agrobacterium tumefaciens* with multiple copies per cell. We designed PCR-based procedures to rapidly generate multiple sgRNA expression cassettes. During our preparation of this article, a strategy for generating multiple target-sgRNAs from linked polycistronic tRNA–gRNA units (each 173 bp) using the endogenous tRNA processing system was reported (Xie et al., 2015). Although the independent sgRNA expression cassettes used in our system have longer lengths (mostly 254–599 bp, one with a length of 894 bp; Supplemental Figure 3), the Golden Gate and Gibson Assembly methods enable the simultaneous delivery of multiple such cassettes (up to eight in this study), with high efficiency, into the binary CRISPR/Cas9 vectors in a single cloning event. If the ligated multiple cassettes are PCR-amplified and then subjected again to the Golden Gate cloning or Gibson Assembly with the binary vectors (see Methods), the cloning efficiency can be increased to clone more sgRNA expression cassettes into the binary vectors. Given the ability of this system to target multiple genomic sites in single binary constructs, designing two target sites for single genes is highly recommended to increase the mutation rate of target genes if the numbers of genes to be targeted are few, especially in *Arabidopsis*, which has relatively lower editing efficiency.

Based on the comprehensive evaluation of large numbers of target sites and editing events, we showed that this system has a high editing efficiency (85.4%) in rice. In addition, with our system it is possible to obtain biallelic or homozygous mutations in *Arabidopsis* T_1 plants. The relatively high editing efficiency of this system may be partly attributed to our modified *Cas9p* gene with plant-optimized codons, including the 5′ enrichment of GC content mimicking the Gramineae genes (Wong et al., 2002), being driven by the strong promoters. Some CRISPR/Cas9 systems for plants directly used the *Cas9* gene originally designed for genome editing in animals (Mao et al., 2013; Xie and Yang, 2013), which works in plant genome targeting but may be suboptimal.

Besides the plant codon optimization of *Cas9*, the CRISPR/Cas9-induced editing efficiency in plants may be affected by several other factors, including the expression levels of *Cas9* and sgRNA, the sequence composition (such as GC content) of targets, and the secondary structure of the target-sgRNAs. Our expression analysis suggests that the expression level of *Cas9* in both rice and *Arabidopsis* may not be the main limiting factor. We further observed that the overall levels of the sgRNA in the *Arabidopsis* transgenic plants were lower than those of the sgRNAs in the eight-target rice transgenic plant (Figure 4), and the rice and *Arabidopsis* transgenic plants with very low sgRNA levels did not produce targeted mutations. Jiang et al. (2014) suggested that the *Cas9* and/or sgRNA genes in transgenic *Arabidopsis* plants may be silenced. Therefore, the relatively low level and even silencing of the sgRNA expression in individual transgenic *Arabidopsis* plants may contribute to the lower editing efficiency compared with that in rice.

We observed that the target sequences with higher GC contents had relatively higher editing efficiencies. Therefore, selection of targets with GC contents of about 50%–70%, and those with less or no base-pairing with the sgRNA sequence, is desirable. Use of target sequences with higher GC contents may potentially lead to a higher risk of off-targeting (Tsai et al., 2015), a critical issue in clinical research. However, for basic and applied research in plants, off-targeting may not be a critical problem, because the risk of off-targeting in plants by the CRISPR/Cas9 system may not be higher than that of the frequent somatic mutations that occur during the tissue culture-based transformation or other mutagenesis treatments. Furthermore, this risk of off-targeting can be minimized by selection of highly specific target sequences by genome searching (Lei et al., 2014; Xie et al., 2014) (see Methods), and unwanted off-targeted mutations in plants can be eliminated, if necessary, by crossing (and backcrossing) the mutant plants with their parental lines.

A previous report showed that CRISPR/Cas9-induced DSB in a mutated gene in mice could be corrected to wild-type through the HR mechanism, also termed homology-directed repair (HDR), with the endogenous wild-type allele as the template (Wu et al., 2013). Based on the HDR mechanism, a mutagenic chain reaction (MCR) strategy was developed to rapidly generate homozygous mutations in insect populations (Gantz

and Bier, 2015). However, the efficiency of this similar HDR mechanism and whether it exists in plants are still unclear. In this study, we detected a high proportion (27.8%) of homozygous mutations in the targeted mutation sites of the T_0 rice plants, much higher than that (7.7%) previously reported in rice (Zhang et al., 2014). It is unlikely that all of the homozygous mutations resulted from independent, identical allelic editing events by the NHEJ mechanism, because the expected frequency of the homozygous mutations by such independent editing events was about 11.7% of the mutated sites. Therefore, we reasoned that the remaining homozygous mutations (16.1%) might have resulted from the following steps: a mutation is produced firstly in a target site of a chromosome via the DSB–NHEJ mechanism. Then the intact allelic target site of another chromosome is cleaved by the Cas9/sgRNA complex and repaired, with the firstly mutated allele as the template, by the HDR mechanism, thereby achieving a homozygous mutation in the site.

This study and other reports (Feng et al., 2014; Zhang et al., 2014) have shown that the editing efficiency and mutation types induced by CRISPR/Cas9 systems are very different in the first transgenic generation of rice and *Arabidopsis*. In T_0 rice the editing efficiency is high and almost all mutations are uniform types (mainly biallelic and homozygous, and a few heterozygous), suggesting that the CRISPR/Cas9-induced editing events take place mainly in the T-DNA transformed callus cells before regeneration. By contrast, in *Arabidopsis* T_1 plants the editing efficiency is relatively low, and both uniform and chimeric mutations were produced. Since the ovules are the primary target of the T-DNA transfer in the *Arabidopsis* infiltration transformation (Desfeux et al., 2000), the targeted editing might occur as early as in the embryo sac cells of the ovules or the female germline cells of the infiltrated plants (T_0), in the zygotes (T_1), and later in the vegetative tissues. However, for both rice and *Arabidopsis* plants, targeted mutagenesis efficiency in the vegetative tissues (other than calli of rice) might be relatively low even though the expression level of *Cas9p* was high (Figure 4); in many targeting cases with heterozygous mutations, most of the unedited alleles (wild-type) were retained intact during the multiple growth stages, although some new mutations were produced (Supplemental Tables 6 and 7). Therefore, the distinct transformation methods and differential editing efficiencies may explain why the proportions of heterozygous and chimeric mutations are much higher in *Arabidopsis* than those in rice.

In this study, we showed the ability of this system to create mutants in T_0 rice and T_1 *Arabidopsis* plants that showed phenotypic variations by simultaneous targeting of multiple members of a gene family, multiple genes in a biosynthetic pathway, or multiple sites in a gene. In addition, using our system other researchers have obtained a high frequency of mutants (T_0) of *Torenia fournieri* with varied floral phenotypes by targeting the *TfTCP* genes, and albino mutants (T_0) of poplar by targeting *PtoPDS* (D. Luo and K. Luo, personal communications). The ability to create loss-of-function gene mutations (i.e., biallelic or homozygous mutations) in the first transgenic generation is particularly important for genome targeting in woody plants.

In summary, we have developed a robust, easy-to-use CRISPR/Cas9 system for highly efficient targeting of multiple genes and genomic sites in monocots and dicots. The facile nature of this system provides a simple and versatile toolbox for studying functions of multiple genes and gene families in plants for basic research and genetic improvement.

METHODS

Design and Synthesis of *Cas9p*, and Construction of the CRISPR/Cas9-Related Vectors

The *Cas9p* gene sequence including the nuclear localization signals was designed with codon optimization for plants, including higher GC content at the 5′ terminal region for Gramineae genes (Wong et al., 2002). This gene sequence was synthesized using multiple rounds of overlapping PCR. Using the Omega-PCR cloning method (Chen et al., 2013), *Cas9p* was linked to the P_{ubi} or P_{35S} promoter in intermediate plasmids, and the cassettes were cloned into binary vectors derived from pCAMBIA1300 (Cambia, Canberra, Australia), which had the *HPT*, *NPT II*, and *Bar* genes, respectively. A *Bsa*I site that originally existed in the pCAMBIA1300 backbone had been destroyed by the Omega-PCR (Chen et al., 2013). A fragment containing a modified *ccdB* flanked by two *Bsa*I sites was cloned into the vectors to produce the CRISPR/Cas9 binary vectors (Figure 1B). *E. coli* strain Top10 F′ was used for maintaining the binary vectors.

The sgRNA sequences were synthesized using overlapping PCR. The OsU3, OsU6b, and OsU6c promoter sequences were amplified from a *japonica* rice cultivar Nipponbare, OsU6a from an *indica* cultivar 93-11, and those of AtU3b, AtU3d, AtU6-1, and AtU6-29 from *Arabidopsis* Columbia. The sgRNA and promoter sequences were linked with in reverse orientation by overlapping PCR, then the fragment was cloned into the pUC18 vector digested with *Bam*HI and *Hin*dIII, to establish the sgRNA intermediate vector sets, which were maintained in *E. coli* DH10B. By Omega-PCR cloning, an *E. coli* promoter–*LacZ* sequence (198 bp in length) was inserted into pYLsgRNA-OsU6a and pYLsgRNA-AtU3b, to produce pYLsgRNA-OsU6a/LacZ and pYLsgRNA-AtU3b/LacZ.

Selection of Target Sequences

Target sequences were selected within the target genes, and a Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the target sequences (including PAM) against the rice or *Arabidopsis* genome sequence was carried out to confirm their targeting specificity in the genomes. Potential target sequences should have a difference of at least two bases compared with similar non-target sequences within the PAM or PAM-proximal region, and those having less than five base mismatches in the PAM-distal region to non-target sequences should not be used as targets (Jinek et al., 2012; Cong et al., 2013; Hsu et al., 2013). Secondary structure analysis of target-sgRNA sequences was carried out with the program RNA Folding Form (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3).

Generation of sgRNA Expression Cassettes with Target Sequences

A restriction–ligation reaction (10 μl) was prepared to contain 1× *Bsa*I reaction buffer (NEB) plus 1.0 mM ATP (or plus 1.0 μl of 10× NEB ligation buffer that contains 10 mM ATP), 5 U of *Bsa*I, ca. 20 U of T4 DNA ligase (Takara, Dalian, China), ca. 20 ng of a pYLgRNA-OsU# (-AtU#) vector, and a target adaptor (0.05 μM). The reactions were incubated in a thermo-cycler for six cycles (37°C, 5 min; 25°C, 5 min). The ligated products (0.5–1 μl) were amplified firstly in 20 μl with 0.2 μM each of U-F and gR-R primers (Supplemental Table 1) using 0.2 U of High-Fidelity DNA polymerase KOD-Plus or KOD FX (Toyobo, Osaka, Japan) for 28 cycles (95°C, 10 s; 58°C, 15 s; 68°C, 20 s). Secondary PCRs (25–30 μl) were set up with 0.2 μl of the first PCR products and combinations of the site-specific (GG) primer pairs (0.2 μM each) for Golden Gate cloning (Supplemental Table 2) or those (GA) for Gibson Assembly (Supplemental Table 3), and run for 15–18 cycles (95°C, 10 s; 60°C, 15 s; 68°C, 30 s).

Alternatively, target sequences were introduced into sgRNA expression cassettes by overlapping PCR (Supplemental Figure 4). The first round of PCR (20 μl) used four primers: U-F and gR-R (0.2 μM each) and two target sequence-containing chimeric primers U#T#+ and U#T#− (0.05–0.1 μM each) (Supplemental Table 1), and 0.2 U of KOD-Plus or KOD FX, for 28 cycles (95°C, 10 s; 58°C, 15 s; 68°C, 20 s). The second PCR was as described above for 25–28 cycles.

Cloning of Single and Multiple sgRNA Expression Cassettes in the CRSPR/Cas9 Binary Vectors

For Golden Gate cloning, restriction–ligation reactions (15 μl) were set up with 1× *Bsa*I reaction buffer plus 1.0 mM ATP (or 1.5 μl of NEB 10× ligation buffer), 10 U of *Bsa*I, 35 U of T4 DNA ligase (Takara, China), 60–80 ng of the intact binary plasmid (pYLCRISPR/Cas9P$_{ubi}$-H or pYLCRISPR/Cas9P$_{35S}$-H), and the purified PCR products (15 ng for each sgRNA expression cassette) amplified with the GG primers. The reactions were incubated for 10–15 cycles (95°C, 10 s; 60°C, 15 s; 68°C, 20 s).

For Gibson Assembly cloning, home-made 2× isothermal *in vitro* recombination master mixture was prepared as previously described (Jiang et al., 2013a, 2013b). Purified PCR products (15 ng for each sgRNA expression cassette) amplified with the GA primers and 60–80 ng of linearized pYLCRISPR/Cas9P$_{ubi}$-H or pYLCRISPR/Cas9P$_{35S}$-H plasmid digested with *Bsa*I were mixed in a total volume of 8 μl. An equal volume of the assembly master mixture was added, and the reaction was incubated at 50°C for 30 min.

The ligated products with multiple sgRNA expression cassettes (up to eight) were directly used to transform *E. coli* competent cells. However, in cases with five or more sgRNA expression cassettes and with the reactions not optimally set, the ligation efficiency may be relatively poor. Therefore, as an optional step the ligated products (including the binary vector) with either method could be used as templates to amplify the linked sgRNA expression cassettes using two flanking primers, PB-L and PB-R (Figure 2A and Supplemental Table 1). A PCR (30–40 μl) with 0.5 μl of the ligated product and 0.5 U of KOD FX, which is suitable for amplification of longer DNA, was set up and thermal cycling was started. When the temperature reached 80°C or higher, PB-L and PB-R primers were added to each final concentration of 0.2 μM. Then 10 cycles (97°C, 10 s; 60°C, 20 s; 68°C, 3–5 min [using 1 min for about 1 kb of the PCR products]) and a subsequent 15 cycles (97°C, 10 s; 68°C, 3–5 min) were run. Specific PCR product was purified from agarose gel using a purification kit (GenStar, Fuzhou, China; cat. no. D205-01), and 40–50 ng of the PCR product and 60–80 ng of the intact binary plasmid were subjected to restriction–ligation as described above.

The ligated products were desalted by dialysis in Millipore VSWP04700 against 0.3× TE buffer for about 30 min (for Golden Gate ligation) or about 15 min (for Gibson Assembly), and 1 μl of desalted products was added to 20 μl of *E. coli* DH10B competent cells for electroporation at 1500–1600 V/mm using the GenePulser Xcell (Bio-Rad, Hercules, CA, USA). Positive colonies on an LB-agar medium plate containing 25 μg/ml of kanamycin plus 40 μl of X-gal (20 mg/ml) were selected for further analysis by PCR, *Mlu*I or *Asc*I digestion, and sequencing.

Plant Transformation

The CRISPR/Cas9 constructs were introduced into *A. tumefaciens* strain EHA105 by electroporation. Transformation of rice and *Arabidopsis* was performed as described previously (Hiei et al., 1994; Clough and Bent, 1998).

Mutation Detection

Genomic DNA extraction from leaves of transgenic rice plants, and rosette leaves of transgenic *Arabidopsis* plants, was carried out using the sodium dodecyl sulfate method (Dellaporta et al., 1983). In some cases genomic DNAs from very small amounts of leaves (1–5 mg) were prepared by breaking the leaf pieces in 0.5× TE as described previously (Wang et al., 2013a, 2013b, 2013c), and the solution (0.5 μl) was directly used for PCR.

PCR amplifications were carried out using primer pairs flanking the designed target sites. The PCR products (ca. 400–600 bp) were sequenced directly using internal specific primers, of which the binding positions are desirably at about 150–250 bp upstream of the target sites. Some samples were cloned into a plasmid vector, and five to six clones for each sample were sequenced. Heterozygous and biallelic mutations that produced superimposed sequence chromatograms from direct sequencing were decoded using our Degenerate Sequence Decoding method (Ma et al., 2015).

RT–qPCR Analysis

Total RNA was extracted from rice calli induced from T_1 seeds, and leaves of *Arabidopsis* T_1 plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcriptions for *Cas9p*, *OsActin 1*, *AtActin 2*, and the target-sgRNAs were carried out with 1 μg of RNA using a mixture of reverse primers specific to the genes and sgRNA, and qPCRs were performed with the gene-specific and target-specific primers (Supplemental Table 8).

Measurement of Amylose Content

Amylose contents of rice seeds were measured as described previously (Perez and Juliano, 1978), with three replicates for each sample.

ACCESSION NUMBERS

Sequence data from this article can be found in the GenBank data library under accession numbers GeneBank: KR029097, KR029098, KR029099, KR029100, KR029101, KR029102, KR029103, KR029104, KR029105, KR029106, KR029107, KR029108, KR559259, KR559260 for the sgRNA intermediate plasmids, and GeneBank: KR029109, KR029110, KR029111, KR029112, KR029113 for the CRISPR/Cas9 binary vectors.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

This work was supported by grants from Ministry of Agriculture of the People's Republic of China (2014ZX08010-001, 2014ZX08009002).

ACKNOWLEDGMENTS

We thank Danhua Zhou (South China Agricultural University) for assistance with measurement of amylose content and Dr. Jian-Feng Li (Sun Yat-sen University) for commenting on the manuscript. No conflict of interest declared.

Received: March 8, 2015
Revised: April 10, 2015
Accepted: April 15, 2015
Published: April 23, 2015

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