revision process of this work, an independent study reported the simultaneous generation of multiple mutations in mice[7]. Our work, together with the mice work, demonstrates that it should be feasible to produce gene-targeted models in rodents and probably other mammalian species using the CRISPR-Cas systems.

Note: Supplementary information is available in the online version of the paper (doi/10.1038/nbt.2652).

ACKNOWLEDGMENTS
This study was supported by grants from the National Basic Research Program of China 2012CBA01300, "Strategic Priority Research Program" of the Chinese Academy of Sciences XDA01020101, the National Science Foundation of China 90919060 and the Ministry of Science and Technology of China 2011CBA01101 (all to Q.Z.).

AUTHOR CONTRIBUTIONS
Q.Z. designed the experiments, supervised laboratory work, analyzed and interpreted data; Q.Z. and W.L. wrote the paper; W.L., F.T. and T.L. performed the experiments.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Wei Li[1,3], Fei Teng[1–3], Tianda Li[1] & Qi Zhou[1]

[1]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China. [2]University of Chinese Academy of Sciences, Beijing, China. [3]These authors contributed equally to this work.
e-mail: qzhou@ioz.ac.cn

1. Jinek, M. *et al. Science* **337**, 816–821 (2012).
2. Cho, S.W., Kim, S., Kim, J.M. & Kim, J.S. *Nat. Biotechnol.* **31**, 230–232 (2013).
3. Cong, L. *et al. Science* **339**, 819–823 (2013).
4. Mali, P. *et al. Science* **339**, 823–826 (2013).
5. Hwang, W.Y. *et al. Nat. Biotechnol.* **31**, 227–229 (2013).
6. Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L.A. *Nat. Biotechnol.* **31**, 233–239 (2013).
7. Wang, H. *et al. Cell* **153**, 910–918 (2013).
8. Geurts, A.M. *et al. Science* **325**, 433 (2009).
9. Tong, C., Li, P., Wu, N.L., Yan, Y. & Ying, Q.L. *Nature* **467**, 211–213 (2010).
10. Tesson, L. *et al. Nat. Biotechnol.* **29**, 695–696 (2011).
11. Wu, H. & Zhang, Y. *Genes Dev.* **25**, 2436–2452 (2011).
12. Gu, T.P. *et al. Nature* **477**, 606–610 (2011).
13. Dawlaty, M.M. *et al. Dev. Cell* **24**, 310–323 (2013).

Targeted genome modification of crop plants using a CRISPR-Cas system

To the Editor:

Although genome editing technologies using zinc finger nucleases (ZFNs)[1] and transcription activator-like effector nucleases (TALENs)[2] can generate genome modifications, new technologies that are robust, affordable and easy to engineer are needed. Recent advances in the study of the prokaryotic adaptive immune system, involving type II clustered, regularly interspaced, short palindromic repeats (CRISPR), provide an alternative genome editing strategy[3]. Type II CRISPR systems are widespread in bacteria; they use a single endonuclease, a CRISPR-associated protein Cas9, to provide a defense against invading viral and plasmid DNAs[4]. Cas9 can form a complex with a synthetic single-guide RNA (sgRNA), consisting of a fusion of CRISPR RNA (crRNA) and *trans*-activating crRNA. The sgRNA guides Cas9 to recognize and cleave target DNA. Cas9 has a HNH nuclease domain and a RuvC-like domain; each cleaves one strand of a double-stranded DNA. It can be used as an RNA-guided endonuclease to perform sequence-specific genome editing in bacteria, human cells, zebrafish and mice[5–11]. Here we show that customizable sgRNAs can direct Cas9 to induce sequence-specific genome modifications in the two most widely cultivated food crops, rice (*Oryza sativa*) and common wheat (*Triticum aestivum*).

We first codon-optimized *Streptococcus pyogenes Cas9* (*SpCas9*), attached nuclear localization signals (NLSs) at both ends (**Fig. 1a** and **Supplementary Fig. 5**) and expressed sgRNA transcripts (**Fig. 1a**, **Supplementary Methods** and **Supplementary Fig. 4**). To disrupt endogenous genes in rice protoplasts, we designed two sgRNA, SP1 and SP2, which target different DNA strands of the rice phytoene desaturase gene *OsPDS* (**Fig. 1b** and **Supplementary Table 4**). Efficient, targeted mutagenesis (15%) was detected starting at 18 h of protoplast cultivation, and similar, if not higher, efficiencies were observed from 24 h through 72 h (**Supplementary Fig. 1a**,**b**). PCR/restriction enzyme (PCR/RE) assays were carried out to detect mutations in both target regions (**Supplementary Methods** and **Supplementary Table 5**). Digestion-resistant bands were detected in both sgRNA targets with efficiencies ranging from 14.5% to 20.0%, as estimated by band intensities (**Fig. 1c** and **Supplementary Methods**). Cloning and sequencing of these uncut bands revealed indels in the targeted *OsPDS* gene. The highest frequency of mutations was obtained with an sgRNA with 20 nucleotides (nts) of sequence complementary to the *OsPDS*-SP1 target site ($P = 0.039$) (**Supplementary Fig. 1c**,**d**).

We targeted another three rice genes (*OsBADH2*, Os02g23823 and *OsMPK2*) and one wheat gene (*TaMLO*) (**Supplementary Tables 1** and **4**) in protoplasts, with indel frequencies of 26.5–38.0% (**Supplementary Fig. 2** and **Supplementary Table 2**). The frequency of mutations induced by sgRNA:Cas9 in Os02g23823 was lower (26.0%) than that induced by TALENs (36.5%) (**Supplementary Fig. 1e**), whereas, in *OsBADH2*, it was considerably higher[12] (26.5% versus 8.0%) (**Supplementary Fig. 2** and **Supplementary Table 7**). Our results suggest that a customized sgRNA:Cas9 efficiently induces sequence-specific modifications in plants. Moreover, only a single customized sgRNA, encoded by a sequence of ~100 nt, is required to target a specific sequence, and Cas9 does not have to be reengineered for each new target site. The sgRNA:Cas9 system is therefore much more straightforward than ZFNs or TALENs.

To test whether sgRNA:Cas9 can induce gene knockouts in rice plants, we bombarded rice callus cells with Cas9 plasmid and sgRNA expression plasmids designed to cleave either *OsPDS* or *OsBADH2* (**Supplementary Methods**). Transformed, hygromycin-tolerant calli were grown into whole plants. Mutations in *OsPDS*-SP1 were identified in 9 of 96 independent transgenic plants (9.4%), and mutations in *OsBADH2* in 7 of 98 transgenic plants (7.1%) (**Fig. 1d** and **Supplementary Table 2**). In addition, biallelic mutations were identified in 3 of the 9 plants mutated in *OsPDS*-SP1. Two of them were homozygous for the same one-nucleotide insertion (**Fig. 1d**), and all three had the albino and dwarf phenotype (**Fig. 1e**), showing that the rice phytoene desaturase gene had been disrupted.

To examine homology-directed repair (HDR), we designed a single-stranded oligo with a KpnI + EcoRI site to be introduced into *OsPDS* (**Fig. 2a** and **Supplementary Table 6**). To detect such mutations, we used a PCR/RE assay that preferentially amplifies mutated DNA sequences. Protoplast genomic DNA was cleaved with PstI before PCR amplification to enrich for sgRNA:Cas9-induced mutations.

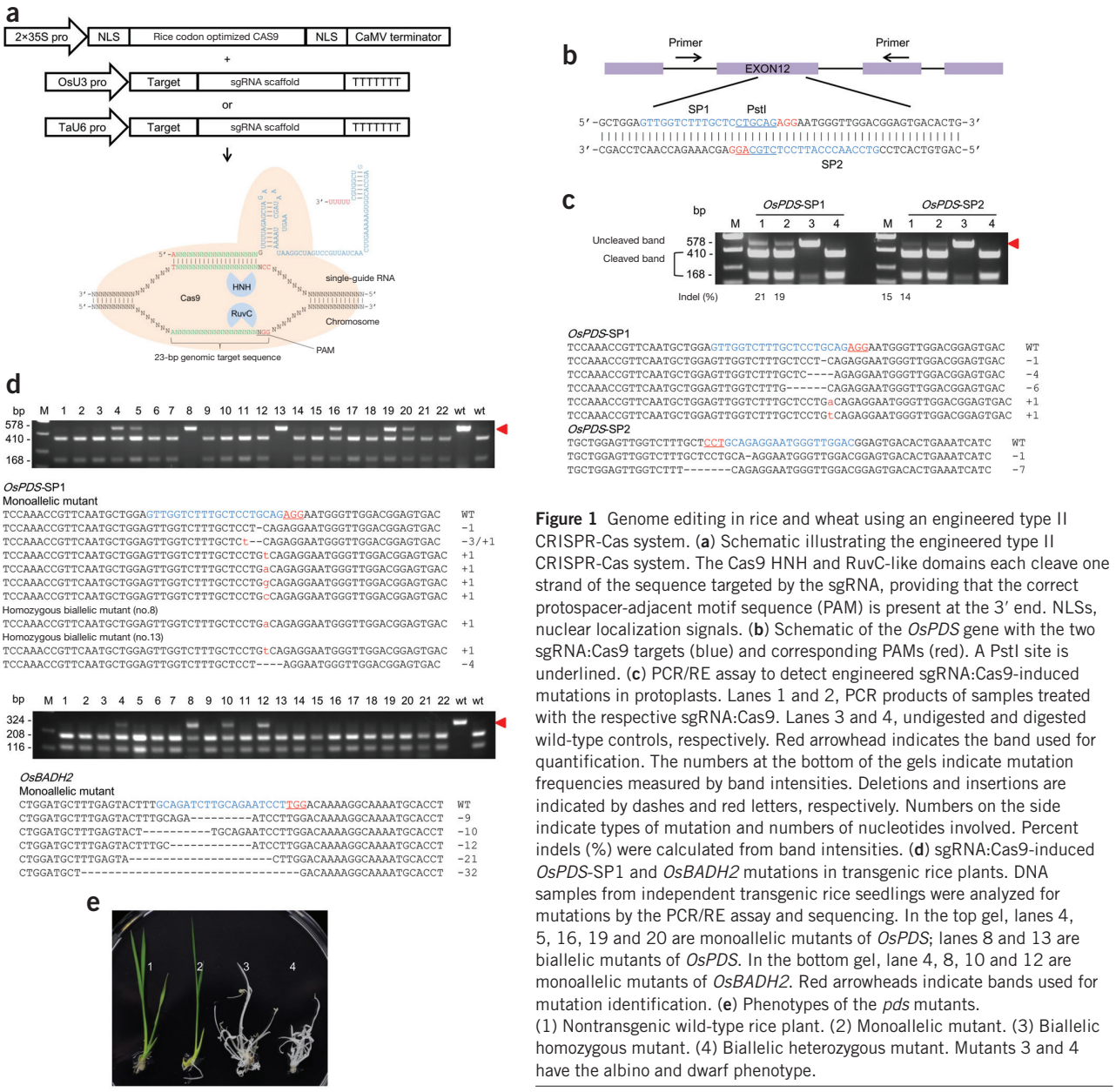

Figure 1 Genome editing in rice and wheat using an engineered type II CRISPR-Cas system. (**a**) Schematic illustrating the engineered type II CRISPR-Cas system. The Cas9 HNH and RuvC-like domains each cleave one strand of the sequence targeted by the sgRNA, providing that the correct protospacer-adjacent motif sequence (PAM) is present at the 3′ end. NLSs, nuclear localization signals. (**b**) Schematic of the *OsPDS* gene with the two sgRNA:Cas9 targets (blue) and corresponding PAMs (red). A PstI site is underlined. (**c**) PCR/RE assay to detect engineered sgRNA:Cas9-induced mutations in protoplasts. Lanes 1 and 2, PCR products of samples treated with the respective sgRNA:Cas9. Lanes 3 and 4, undigested and digested wild-type controls, respectively. Red arrowhead indicates the band used for quantification. The numbers at the bottom of the gels indicate mutation frequencies measured by band intensities. Deletions and insertions are indicated by dashes and red letters, respectively. Numbers on the side indicate types of mutation and numbers of nucleotides involved. Percent indels (%) were calculated from band intensities. (**d**) sgRNA:Cas9-induced *OsPDS*-SP1 and *OsBADH2* mutations in transgenic rice plants. DNA samples from independent transgenic rice seedlings were analyzed for mutations by the PCR/RE assay and sequencing. In the top gel, lanes 4, 5, 16, 19 and 20 are monoallelic mutants of *OsPDS*; lanes 8 and 13 are biallelic mutants of *OsPDS*. In the bottom gel, lane 4, 8, 10 and 12 are monoallelic mutants of *OsBADH2*. Red arrowheads indicate bands used for mutation identification. (**e**) Phenotypes of the *pds* mutants. (1) Nontransgenic wild-type rice plant. (2) Monoallelic mutant. (3) Biallelic homozygous mutant. (4) Biallelic heterozygous mutant. Mutants 3 and 4 have the albino and dwarf phenotype.

PCR products were verified by cloning, restriction digestion with KpnI or EcoRI, and DNA sequencing. Two of 29 single colonies had the expected insertion of the KpnI + EcoRI site into *OsPDS* (**Fig. 2b–d**), demonstrating the possibility of HDR-mediated genome modification by co-transformation of Cas9, sgRNA and single-stranded DNA oligos into plant cells.

We next evaluated potential off-target effects of two sgRNA:Cas9 constructs targeting the *OsMPK2* or *OsPDS* genes. Three nearly identical sequences, PDS_NI-1, MPK2_NI-1 and MPK2_NI-2, with one-base or three-base mismatches to the PDS-SP1 and MPK2 target sites were identified from the rice genome (**Supplementary Fig. 3a**). Using the PCR/RE assay (**Supplementary Table 5**), no evidence of sgRNA:Cas9-induced mutation was found in PDS_NI-1 and MPK2_NI-2. In contrast, several deletion events were identified from the MPK2 target site to the MPK2_NI-1 site, 30 bp downstream of the MPK2 target site (**Supplementary Fig. 3b**). These deletion events could be the products of rejoining broken ends generated by separate cleavages at both the MPK2_NI-1 and MPK2 target sites, suggesting that off-target cleavage can occur in homologous sequences[13]. They could also be explained by homologous recombination between the nearly identical sites induced by a single sgRNA:Cas9-mediated cleavage in the target site. Comprehensive studies using genome-wide approaches are required to thoroughly address the off-target issue for the sgRNA:Cas9 system.

The system described can in principle target any sequences, such as 5′-A-$N_{(20)}$-GG-3′ and 5′-G-$N_{(20)}$-GG-3′ in rice and wheat, respectively. Use of the rice U3 promoter and the wheat U6 promoter constrains the first positions in the corresponding RNA transcripts to be ‘A’ and ‘G’, respectively. A computer search generated ~3,183,497 and ~566,367 sequences specifically targetable by sgRNAs in the rice genome and rice cDNAs, respectively, representing nearly nine targets per cDNA (**Supplementary Table 3**). Loosening these constraints to target

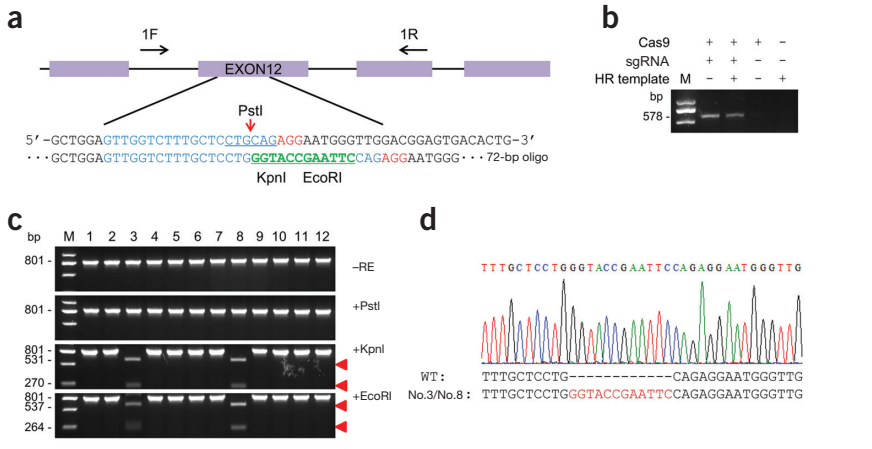

Figure 2 HDR-mediated genome modification in rice protoplasts. (**a**) Schematic of the oligo targeting site in *OsPDS*. The sgRNA targeting sequence is in blue, and the PAM sequence in red. The 72-bp donor oligo is shown under the target site, with 12-bp insertions (KpnI + EcoRI site) in green. (**b**) PCR amplification of the protoplast genomic DNA predigested with PstI to enrich for sgRNA:Cas9-induced mutations. Specific 1F and 1R primers were used. HR, homologous recombination. (**c**) Targeted integration of the KpnI and EcoRI restriction sites. The enrichment PCR product (+Cas9, +sgRNA, +HR template) was cloned into pEASY-Blunt vector (TransGen). Lanes 1–12, representative PCR products of cloned alleles for digesting assay; –RE, PCR amplification of colonies with M13F/R primers; +PstI, +KpnI and +EcoRI, PCR products digested with PstI, KpnI and EcoRI, respectively. Two cloned alleles (no. 3 and no. 8, arrowhead) with KpnI and EcoRI insertions were identified. (**d**) Sanger sequencing results for cloned alleles no. 3 and no. 8 show HDR-mediated targeting. Inserted sequences are labeled in red.

sequences of the form 5′-A-$N_{(19–21)}$-GG-3′ identified 32 targets on average per cDNA. The wheat A and D genomes yielded similar results (**Supplementary Table 3**). Our findings establish that the sgRNA:Cas9 system can be used for rice and wheat genome modification, the first plants shown to be amenable to this gene editing approach.

Note: Supplementary information is available in the online version of the paper (doi:10.1038/nbt.2650).

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (201263, 383601 and 31200273), the Ministry of Agriculture of China (2011ZX08002-004 and 2013ZX08010-002) and Chinese Academy of Sciences (KSCX2-EW-N-06, KSCX2-EW-J-6). We thank X. Wang for bioinformatics analysis.

AUTHOR CONTRIBUTIONS

Q.S., Y.W., J.L., Y.Z., K.C., Z.L., J.J.X., J.-L.Q. and C.G. designed the experiments; Q.S., Y.W., J.L., Y.Z., K.Z. and J.L. performed experiments; Q.S., Y.W., J.L., J.-L.Q. and C.G. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Qiwei Shan[1,4], Yanpeng Wang[1,4], Jun Li[1,4], Yi Zhang[1], Kunling Chen[1], Zhen Liang[1], Kang Zhang[1], Jinxing Liu[1], Jianzhong Jeff Xi[2], Jin-Long Qiu[3] & Caixia Gao[1]

[1]State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. [2]Institute of Molecular Medicine, Peking University, Beijing, China. [3]State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. [4]These authors contributed equally to this work.
e-mail: cxgao@genetics.ac.cn or qiujl@im.ac.cn

1. Zhang, F. *et al. Proc. Natl. Acad. Sci. USA* **107**, 12028–12033 (2010).
2. Chen, K. & Gao, C. *J. Genet. Genomics* **40**, 271–279 (2013).
3. Mussolino, C. & Cathomen, T. *Nat. Biotechnol.* **31**, 208–209 (2013).
4. Barrangou, R. *et al. Science* **315**, 1709–1712 (2007).
5. Jinek, M. *et al. Science* **337**, 816–821 (2012).
6. Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. *Proc. Natl. Acad. Sci. USA* **109**, E2579–E2586 (2012).
7. Cong, L. *et al. Science* **339**, 819–823 (2013).
8. Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L.A. *Nat. Biotechnol.* **31**, 233–239 (2013).
9. Hwang, W.Y. *et al. Nat. Biotechnol.* **31**, 227–229 (2013).
10. Chang, N. *et al. Cell Res.* **23**, 465–472 (2013).
11. Wang, H. *et al. Cell* **153**, 910–918 (2013).
12. Shan, Q. *et al. Mol. Plant* **6**, 1365–1368 (2013).
13. Fu, Y. *et al. Nat. Biotechnol.* advance online publication, doi:10.1038/nbt.2623 (23 June 2013).

Multiplex and homologous recombination–mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9

To the Editor:

Elucidation and manipulation of human, animal and plant genomes is key to basic biology research, medical advances and crop improvement. The development of targeted genome editing, particularly homologous recombination–based gene replacement, is of great value in all organisms. Recent advances in engineered nucleases with programmable DNA-binding specificities, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have provided valuable means to create targeted mutations in metazoan and plant genomes with high specificity[1–6]. However, these technologies demand elaborate design and assembly of individual DNA-binding proteins for each DNA target site[1–6]. Recently, a simple, versatile and efficient genome engineering technology has been developed based on the bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated protein (Cas) adaptive immune systems[7]. In a type II CRISPR-Cas system from *Streptococcus pyogenes*, a single Cas9 endonuclease guided by a duplex of mature CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA) cleaves trespassing DNA from bacteriophage or plasmids in a sequence-specific manner[7]. By reconstitution of the *S. pyogenes* Cas9 (SpCas9) and an artificial chimera of crRNA and tracrRNA called synthetic-guide RNA (sgRNA) in eukaryotic cells, including yeast, zebrafish, mouse and human cells, targeted genome editing has been achieved through either error-prone nonhomologous end joining (NHEJ) or homology-directed repair (HDR) of the intended cleavage site[7–14]. Here, we show the feasibility and efficacy of sgRNA:Cas9–based genome editing