Genome editing in rice and wheat using the CRISPR/Cas system

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Targeted genome editing nucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), are powerful tools for understanding gene function and for developing valuable new traits in plants. The clustered regularly interspersed short palindromic repeats (CRISPR)/Cas system has recently emerged as an alternative nuclease-based method for efficient and versatile genome engineering. In this system, only the 20-nt targeting sequence within the single-guide RNA (sgRNA) needs to be changed to target different genes. The simplicity of the cloning strategy and the few limitations on potential target sites make the CRISPR/Cas system very appealing. Here we describe a stepwise protocol for the selection of target sites, as well as the design, construction, verification and use of sgRNAs for sequence-specific CRISPR/Cas-mediated mutagenesis and gene targeting in rice and wheat. The CRISPR/Cas system provides a straightforward method for rapid gene targeting within 1–2 weeks in protoplasts, and mutated rice plants can be generated within 13–17 weeks.

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INTRODUCTION

In recent years, genome editing technologies, ZFNs[1–3] and TALENs[4–8] have been used to mutate specific loci in various plants[9–16]. Both ZFNs and TALENs are fusion proteins that consist of a DNA-binding domain fused to the FokI endonuclease domain. The endonuclease domain induces DNA double-strand breaks (DSBs) at targeted sites in the genome, which are determined by the binding specificity of the DNA-binding domain. These targeted DSBs can be repaired by either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR)[17]. NHEJ is often imprecise and frequently introduces small deletions or insertions at the junction of the newly rejoined chromosome. If the sequence change causes a frameshift mutation or a premature stop codon in the target gene product, a knockout (loss-of-function) mutation is created. HDR is an alternative means of repairing a broken chromosome. HDR is stimulated by the homologous DNA template surrounding a DSB, and it is a precise gene targeting method.

Recently, an RNA-guided genome editing system has been described: CRISPR/Cas[18–20]. Similarly to ZFNs and TALENs, the CRISPR/Cas system generates targeted DSBs that are then repaired by NHEJ or HDR (**Fig. 1a**). The CRISPR/Cas system has already been widely used to perform precise genome editing in a great range of organisms, including human cells, bacteria, yeast, *Caenorhabditis elegans*, zebrafish, *Drosophila*, mice, rat, *Arabidopsis*, tobacco, rice, wheat, maize and sorghum[16,21–36]. We reported previously that the CRISPR/Cas system can be used to induce sequence-specific genome modifications in the two most widely cultivated food crop plants: rice and wheat[24]. Here we provide an improved procedure for rapid construction of customized sgRNAs and methods to apply these sgRNAs to achieve targeted mutagenesis and gene targeting in rice and wheat. We have successfully generated rice and wheat protoplasts via NHEJ- or HDR-mediated gene targeting, and stable rice plants via NHEJ-mediated gene targeting.

The CRISPR/Cas system

The CRISPR/Cas system evolved as an adaptive immune response in bacteria and archaea to defend against invading viral and plasmid DNAs[37–39]. Three types of CRISPR systems (types I–III) have been identified[39–42], but it is the type II system from *Streptococcus pyogenes* that is best characterized and that has been adapted for gene targeting purposes. This system consists of a single gene encoding the Cas9 protein and two RNAs, a mature CRISPR RNA (crRNA) and a partially complementary trans-activating crRNA (tracrRNA). crRNA hybridizes with tracrRNA, and these two RNAs complex with the Cas9 protein to cleave complementary target-DNA sequences, if they are adjacent to short sequences known as protospacer-adjacent motifs (PAMs). The crRNA-tracrRNA heteroduplex can be fused to generate a chimeric, sgRNA containing a designed hairpin[18] (**Fig. 1b**). For the CRISPR/Cas system from *S. pyogenes*, the 20-bp DNA target must lie immediately 5′ of a PAM sequence that matches the canonical form 5′-NGG (ref. 43). Thus, Cas9 nuclease can be targeted to any DNA sequence of the form 5′-$N_{(20)}$-NGG simply by changing the first 20-nt guide sequence within the sgRNA. Cas9 has two conserved nuclease domains: an HNH nuclease domain and a RuvC-like nuclease domain. The Cas9 HNH nuclease domain cleaves the strand complementary to the crRNA, whereas the Cas9 RuvC-like nuclease domain cleaves the noncomplementary strand (**Fig. 1b**).

Advantages of the CRISPR/Cas system

The CRISPR/Cas system creates DNA DSBs at target loci to stimulate genome editing via NHEJ or HDR, just like other engineered nuclease technologies such as ZFNs and TALENs. Nevertheless, it possesses several potential advantages over ZFNs and TALENs.

- *Range of target sites.* ZFNs are limited by the range of targetable sequences because of the absence of fingers for all possible

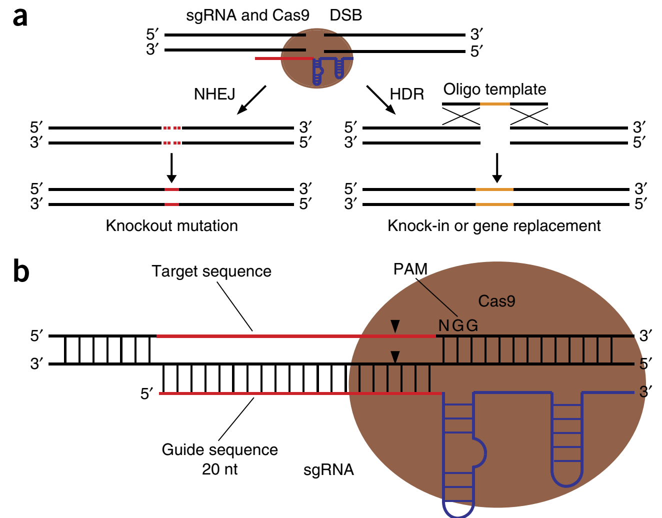

Figure 1 | Schematic description of RNA-guided genome editing using the CRISPR/Cas system. (**a**) DSB repair promotes gene editing. DSBs induced by Cas9 (brown) trigger the DNA repair pathways NHEJ and HDR. The NHEJ pathway is often imprecise and frequently introduces small deletions and insertions at the junction of the newly rejoined chromosome. This can result in a frameshift or premature stop codon generating gene knockout mutations. Alternatively, in the presence of a homologous ssDNA spanning the DSB, the HDR repair pathway can be activated, and a targeted gene knock-in or replacement can result. (**b**) Diagrams illustrating the CRISPR/Cas system composed of Cas9 (brown) and sgRNA (red and blue). The secondary structure of sgRNA mimics that of the crRNA-tracrRNA heteroduplex. The Cas9 HNH and RuvC-like domains each cleave one strand of the sequence targeted by the sgRNA, provided that the correct protospacer-adjacent motif sequence (PAM) is present at the 3′ end.

DNA triplets[44]. In the CRISPR/Cas system, the only requirement for the target site is the 20-bp target sequence preceding a 5′-NGG PAM.

- *Delivery into cells.* The short length of the sgRNA sequence makes it easier to deliver it into cells than the longer and highly repetitive ZFN/TALEN-encoding vectors.
- *Engineering.* For a single target site, two different ZFN and TALEN proteins must be engineered, each consisting of many repetitive ZF and TALE modules, and their construction is time-consuming and expensive. By contrast, the CRISPR/Cas system is an RNA-guided genome editing method, so that Cas9 protein does not require reengineering for each new target site. Once a target site is selected, only one cloning step is required to generate the final constructs carrying sgRNAs. Therefore, the CRISPR/Cas system is much easier to engineer than ZFNs or TALENs.
- *Multiplexing.* Given that the targeting specificity of the CRISPR/Cas system is only dependent on sgRNAs, which are encoded by short sequences of ~100 bp, it is possible to achieve simultaneous multiplex gene editing of plant loci by co-transforming multiple sgRNAs.

Limitations of the CRISPR/Cas system

There are a few potential limitations of the CRISPR/Cas system for genome editing.

- *PAM sequences.* A 5′-NGG PAM sequence is required downstream of target sites for CRISPR/Cas-induced cleavage, which may limit the range of available targets. However, it has been shown that some Cas9 homologs from other strains of archaea or bacteria use different PAMs, so it should be possible to eliminate this constraint by developing different CRISPR/Cas systems using different PAMs.
- *Off-target mutagenesis.* This may occur as a result of targeting homologous sequences in unintended loci[45–47]. To minimize off-target effects, it is necessary to monitor the genome-wide presence of such target sequences and to avoid selecting target sequences with homology to many other sites.
- *Recalcitrant sgRNA/target.* Certain sgRNAs may have low efficiencies or may even fail to work, possibly owing to the chromatin states of target loci, unwanted hairpin structures of sgRNA or other unknown factors.

Experimental design

The general workflow for sgRNA construction, verification and use of the sgRNA and Cas9 for sequence-specific mutagenesis and gene targeting in rice and wheat is summarized in **Figure 2**.

Selecting Cas9 target sites. A 20-nt guide sequence within the sgRNA directs Cas9 to the desired site via Watson-Crick base pairing. In general, three elements influencing Cas9 target site selection should be considered:

- The 20-bp target sequence should immediately precede the 5′-NGG PAM, which is essential for Cas9 binding to the target DNA.
- If the aim is to disrupt gene function, target sequences at the 3′ end of coding regions or introns should be avoided. Gene disruptions in these positions may have little or no effect on gene function.
- Off-target effects should be minimized; we recommend searching the genome using a BLAST search (http://blast.ncbi.nlm.nih.gov/) for the relevant 22-nt sequence—the 20-nt sgRNA-binding sequence plus the GG in the NGG PAM—to make sure that the target sequence is unique.

Select target sequences by identifying the 23-bp sequence for 5′-$N_{(20)}$-NGG-3′ (template strand targeting), or for 5′-CCN-$N_{(20)}$-3′

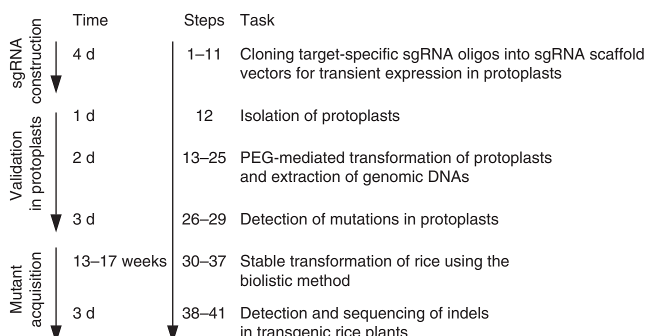

Figure 2 | Timeline for the construction and expression of sgRNAs and Cas9. The steps required for the construction of sgRNA plasmids for transient transformation can be completed in 4 d. Validation in protoplasts takes 6 d (not including the time for plant preparation, we usually prepare plants every 3 d to ensure that there are enough plants for isolating protoplasts at all times). The production of mutant rice plants requires 13–17 weeks.

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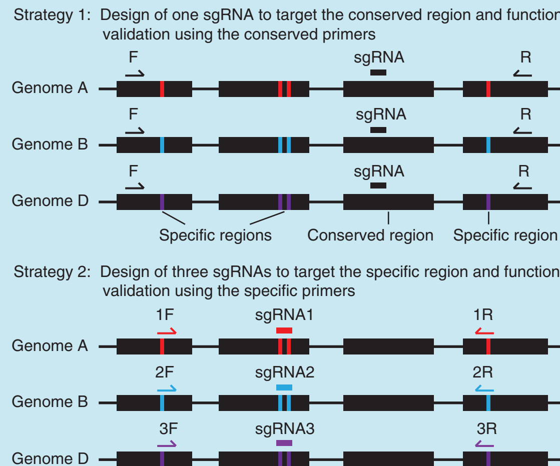
Box 1 | sgRNA design and mutant identification for wheat genome editing

Wheat is a hexaploid species with most genes represented by homologous copies in genomes A, B and D (see illustration in this box), and therefore single gene knockouts in one locus usually fail to cause substantial phenotypic change owing to functional redundancy. Thus, targeting three or even more copies of a gene simultaneously is a challenge for wheat genome editing. The sgRNA in the CRISPR/Cas system is more flexible in terms of target recognition than other available gene editing techniques, such as ZFNs and TALENs, because it can be easily designed to target multiple genes. Here we summarize some key principles for sgRNA design and mutation identification in wheat.



Understanding the background of the targeted gene. Because of the absence of high-quality hexaploid wheat sequence databases and the existence of some polymorphisms for genes in the databases for different varieties, it is usually necessary to clone and sequence target genes to acquire genomic and transcriptomic information about them. On the basis of this sequence information, it is necessary to comprehensively analyze the copy number of target genes and their polymorphisms, in order to design effective sgRNAs to target them.

Designing sgRNAs for targeted wheat genes. There are two approaches to design sgRNAs to target all copies of a gene (see illustration in this box). In the first strategy, using sequence alignment, sgRNAs can be designed to target all the conserved regions. In the second strategy, different sgRNAs can be designed for each copy and co-transformed to target all the copies.

Identifying mutants. There are two methods for detecting mutation, namely the PCR/RE and T7EI assay. If one fails to design specific primers for each copy of the target gene and the targeting region of the sgRNA includes a restriction enzyme site, the PCR/RE assay can be used to detect mutations. In contrast, if all the copies of a target gene can be amplified by specific primers, we recommend using the T7EI assay as the mutation detection method.

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(nontemplate strand targeting). A wheat genome sequence database is not currently available, so some gene cloning and sequencing probably will be needed (**Box 1**). If sgRNA activity will be detected by the PCR/restriction enzyme (PCR/RE) assay (PROCEDURE Step 29A), the restriction enzyme sites within the target sequences at the Cas9 endonuclease cutting site (3-bp upstream 5′-NGG) will facilitate detection. If the T7EI assay (T7 endonuclease I; PROCEDURE Step 29B) will be used, restriction enzyme sites do not need to be considered. Aside from the manual selection of sgRNA target sites, it is worth noting that some web-based tools, such as 'CRISPR-PLANT' (http://www.genome.arizona.edu/crispr/) and 'CasOT' (http://eendb.zfgenetics.org/casot/), have been developed to aid the selection of specific sgRNAs in model plants and major crops (including rice) and to avoid potential off-target sites in any given genome[48,49].

sgRNA scaffold vectors. Only the ~20-nt target sequence of the sgRNA needs to be replaced to target a different genomic site; the remainder of the sgRNA—the 'scaffold'—remains the same. Therefore, we have constructed sgRNA scaffold vectors that allow the target sequences to be easily swapped. We use pOsU3-sgRNA for rice transformation and pTaU6-sgRNA for wheat transformation. Both vectors contain a unique AarI restriction site, into which annealed target oligos can be cloned. AarI is chosen because of its relatively rare recognition site (5′-CACCTGC(4N/8N)-3′). AarI is a type IIS restriction enzyme and requires an oligodeoxyribonucleotide (provided by the supplier) for maximal activity.

Target-specific sgRNA oligos. Once a target site is selected, forward and reverse oligos can be designed and synthesized to insert into the sgRNA scaffold vector (**Fig. 3a**,**b**). The oligos should consist of the 20-nt target site with 5′ and 3′ overhangs complementary to the digested scaffold vector. In our case, these should be AarI-complementary overhangs—which can be any four bases. The OsU3 RNA polymerase III promoter used to express RNAs from the pOsU3-sgRNA vector prefers to start transcripts with an adenine (A) nucleotide, so an A is included immediately 5′ of the target sequence (the fourth of the four additional bases). Similarly, the TaU6 RNA polymerase III promoter prefers a guanine (G) nucleotide as the first base, so a G is included immediately 5′ of the target for inserts cloned into the pTaU6-sgRNA vector. Design and order primers as described in **Figure 3a**,**b**.

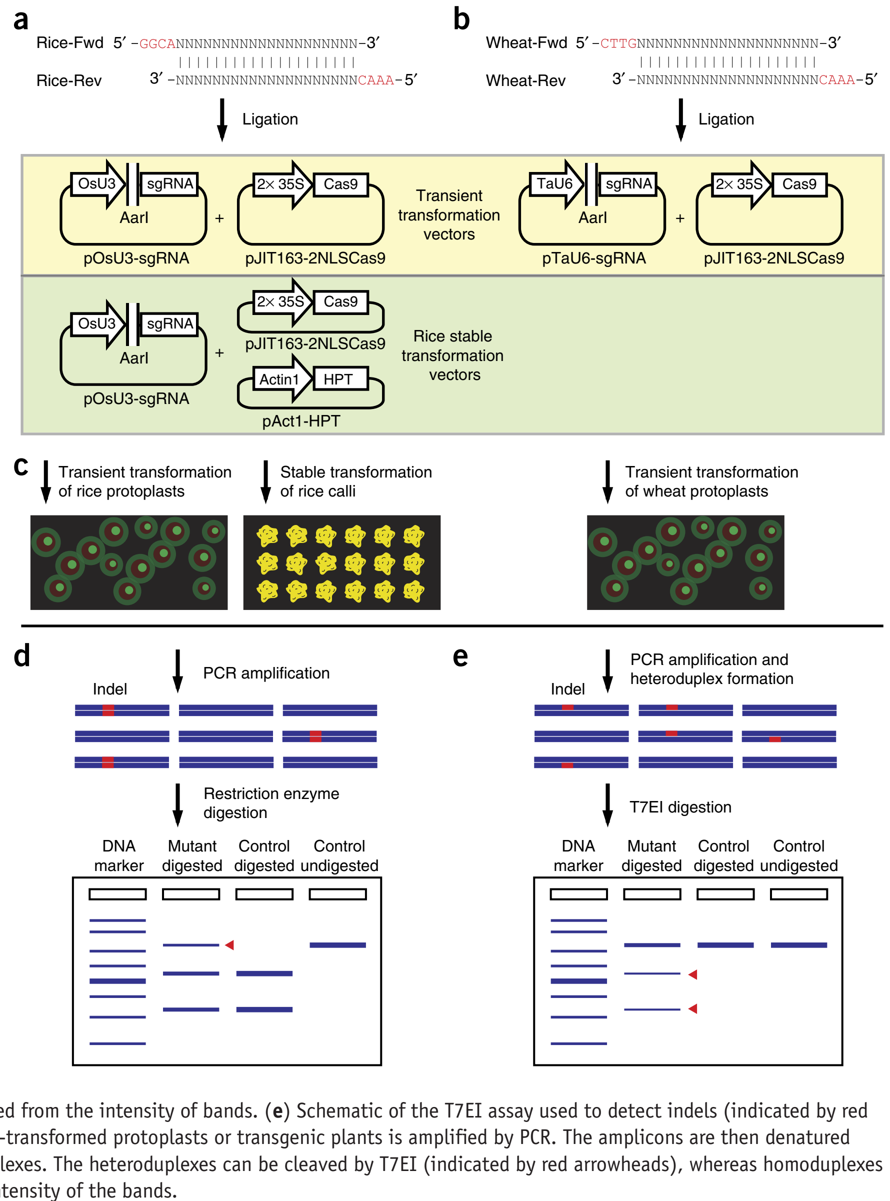

Figure 3 | Overview of the experiments. The steps required for vector construction, transient or stable transformation and validation are illustrated. (**a**) Schematic for cloning the rice guide sequence oligos into a plasmid containing the sgRNA scaffold. The annealed guide sequence oligos contain overhangs (red letters) for ligating them into the pair of AarI sites in the sgRNA vector. The sgRNA plasmid pOsU3-sgRNA co-transformed with the Cas9 expression plasmid pJIT163-2NLSCas9 can be used for transient transformation in rice protoplasts (highlighted in yellow). Co-transforming of pOsU3-sgRNA, pJIT163-2NLSCas9 and pAct1-HPT can be used for stable biolistic transformation of rice calli (highlighted in green). (**b**) Schematic for cloning the wheat guide sequence oligos into a plasmid containing the sgRNA scaffold. The annealed guide sequence oligos contain overhangs (red letters) for ligation into the pair of AarI sites in the sgRNA vector. sgRNA plasmid pTaU6-sgRNA co-transformation with Cas9 expression plasmid pJIT163-2NLSCas9 are used for transient transformation in wheat protoplasts (highlighted in yellow). (**c**) Schematic for transient or stable transformation of sgRNA and Cas9 expression plasmids into protoplasts or other types of recipients. Protoplasts are isolated from rice sheaths and wheat leafs, respectively. Rice calli induced from mature seeds are used for stable transformation. (**d**) Schematic of the PCR/RE assay used to detect indels (indicated by red rectangles). First, genomic DNA from sgRNA- and Cas9-transformed protoplasts or transgenic plants is amplified by PCR. The amplicons are then digested with restriction enzymes that recognize the wild-type target sequences. Mutations introduced by NHEJ are resistant to restriction enzyme digestion because of the loss of the restriction sites, and they result in an uncleaved band (indicated by red arrowhead) in agarose gels. Indel frequency is measured from the intensity of bands. (**e**) Schematic of the T7EI assay used to detect indels (indicated by red rectangles). First, genomic DNA from sgRNA- and Cas9-transformed protoplasts or transgenic plants is amplified by PCR. The amplicons are then denatured and annealed in a thermocycler to generate heteroduplexes. The heteroduplexes can be cleaved by T7EI (indicated by red arrowheads), whereas homoduplexes remain intact. Indel frequency is measured from the intensity of the bands.

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The oligo pairs for rice should be: Rice-Fwd: 5′-GGCAN$_{(20)}$-3′; Rice-Rev: 5′-AAACN$_{(20)}$-3′. For wheat, the oligo pairs should be: Wheat-Fwd: 5′-CTTGN$_{(20)}$-3′; Wheat-Rev: 5′-AAACN$_{(20)}$-3′. For unknown reasons, certain sgRNAs are inefficient or can even fail to work. To obtain efficient sgRNAs and avoid repeating experiments, at least two sgRNAs per target locus should be constructed for rice and wheat. It is worth first testing the efficiencies of sgRNAs in protoplasts.

ssDNA oligos for HDR. DSBs at specific genomic sites can lead to changes at the DNA break sites via HDR if a homologous donor DNA exists. Customarily, the HDR donor is a plasmid or a double-stranded DNA containing long homology arms flanking each side of the target site. Recently, single-stranded DNA oligos (ssDNA, sense or antisense) with shorter homology arms (usually ranging from 20–90-nt long) are being used as donors to introduce small precise changes, including single-base substitution or few-base insertions[50–52]. The proposed site of mutation is usually located at the center of the left and right homology arms[50–52]. We usually ask the oligo supplier to PAGE-purify the ssDNA oligo if it is longer than 40 nt.

Validating sgRNAs by transient expression. To validate and assess the activity of sgRNAs rapidly, pOsU3-sgRNA or pTaU6-sgRNA can be co-transformed with pJIT163-2NLSCas9 into rice or wheat protoplasts for transient expression (**Fig. 3c**). If HDR-mediated genome modifications are required, an ssDNA oligo should also be co-transformed. Genomic DNA can be extracted from transformed protoplasts to identify CRISPR/Cas-induced mutations.

Generating transgenic rice plants by stable transformation. For stable rice transformation, pOsU3-sgRNA, pJIT163-2NLSCas9 and a hygromycin selection marker plasmid pAct1-HPT are co-transformed into rice calli using the biolistic method (**Fig. 3c**). The protocol for rice biolistic transformation is based on previous works[53], with some modifications. Six-to-nine-week-old embryogenic calli of rice cultivar Nipponbare are bombarded

Box 2 | Enrichment of genomic DNA by restriction enzyme digestion ● TIMING 1 h

1. Digest genomic DNA using a restriction enzyme that recognizes the wild-type sgRNA target; mutations introduced by NHEJ and HDR are resistant to restriction enzyme digestion because of the loss of the restriction site, and they result in uncleaved bands. Digest genomic DNA in the following reaction:

Component	Amount (μl)	Final concentration
Fastdigest buffer, 10×	2	1×
MscI, 5 U μl^{-1} (or another enzyme appropriate)	1	5 units
Genomic DNA	17 (30–60 ng μl^{-1})	25–50 ng μl^{-1}
Total	20	

2. Digest the DNA at 37 °C for 1 h.
3. Proceed from Step 26 of the main PROCEDURE.

using a PDS1000/He particle bombardment system. Note that, to date, we have not been able to generate stable rice plants via HDR-mediated gene targeting.

Identifying CRISPR/Cas-induced mutations. CRISPR/Cas-induced mutagenesis usually involves the introduction of small insertions or deletions (indels) into the target sequences. Below we briefly describe three strategies for identifying CRISPR-induced mutations.

- *PCR/RE assay.* A prerequisite of this strategy is that the target locus includes a restriction enzyme site that is destroyed by CRISPR/Cas-induced mutations; the mutant amplicons are therefore resistant to restriction enzyme digestion, and they produce uncleaved bands. PCR primers are designed to amplify a 300–600-bp amplicon that contains the restriction site in or near the middle of the amplicon. Primers should be 20–30 nt long with melting temperatures of ~60°C and should be checked for specific amplification using the National Center for Biotechnology Information (NCBI) Primer-Basic Local Alignment Search Tool (BLAST). The amplicons are then digested with a restriction enzyme that recognizes the wild-type target sequences (**Fig. 3d**), and the indel mutation frequency is estimated from the intensity of the uncleaved band. Subsequently, the mutant alleles can be further characterized by subcloning and sequencing the uncleaved bands.
- *T7EI assay.* The PCR/RE assay cannot be applied to a target locus in which there is no appropriate restriction enzyme site. As an alternative, enzymes that digest mismatched dsDNA such as T7EI or SURVEYOR nuclease can be used[20,28,36]. As for T7EI assays, PCR primers are designed to amplify 300–600 bp surrounding the genomic target site. The PCR products (mixture of wild-type allele and mutant allele) are then denatured and renatured, forming heteroduplexes. The reaction products are digested with T7EI nuclease, and then they are analyzed by 2.0% (wt/vol) agarose gel electrophoresis. The annealed heteroduplexes are cleaved by T7EI, whereas wild-type and mutant homoduplexes are left intact. The indel mutation frequency is estimated from the intensities of the bands (**Fig. 3e**). Subsequently, the mutant alleles can be further identified by subcloning and sequencing.
- *Detection of HDR-mediated insertions by PCR/RE assay and sequencing.* Small precise genomic modifications introduced by ssDNA oligos (sense or antisense) can be detected by PCR/RE assay and sequencing. It should be noted that the frequency of HDR in plants is relatively low. In protoplasts, typically ~1% of treated cells have the desired modification[8]. We recommend enriching the HDR-mediated modification by restriction digestion of protoplast genomic DNA before use in the PCR/RE assay (**Box 2**). Because the restriction sites are destroyed by most NHEJ- and HDR-induced mutations, the mutated sequences are resistant to digestion, and they are amplified preferentially in the subsequent round of PCR. Thereafter, the clonal genotype can be confirmed by subcloning and sequencing.

MATERIALS

REAGENTS

Rice and wheat cultivar

- Rice cultivar: Nipponbare (IGDB, CAS)
- Wheat cultivar: Kenong 199 (IGDB, CAS)

Plasmids

- pOsU3-sgRNA, for sgRNA expression in rice. See **Supplementary Note 1** for the full sequence
- pTaU6-sgRNA, for sgRNA expression in wheat. See **Supplementary Note 2** for the full sequence
- pJIT163-2NLSCas9 for Cas9 expression in rice and wheat. See **Supplementary Note 3** for the full sequence
- pAct1-HPT[24] for Hygromycin selection in rice. See **Supplementary Note 4** for the full sequence
- All plasmids can be obtained from the authors on request ▲ CRITICAL pJIT163-2NLSCas9 and pAct1-HPT should be prepared using a Wizard Plus midiprep kit according to the manufacturer's instructions. The minimum concentration should be 1 μg μl^{-1} with an $A_{260/280}$ ratio range of 1.7–1.9.

sgRNA cloning

- Custom forward and reverse target-specific sgRNA oligos (BGI) designed as described in Experimental design (**Table 1**). See **Supplementary Table 1** for the sequences of oligos used to generate the data shown in **Figure 4** and **Supplementary Table 2**
- Generic PCR/sequencing primers for sgRNA vector verification (BGI). Sequences are listed in **Table 1**. See **Supplemenary Table 3** for the PCR and sequencing primers used to generate the data shown in **Figure 4** and **Supplementary Table 2**.
- pEASY-Blunt cloning vector (TransGen Biotech, cat. no. CB101-01)
- AarI (Fermentas/Thermo Scientific, cat. no. ER1582)
- Buffer AarI, 10× (Fermentas/Thermo Scientific, supplied with AarI)
- Oligonucletide, 50× (Fermentas/Thermo Scientific, supplied with AarI)
- T4 DNA ligase (Fermentas/Thermo Scientific, cat. no. EL0011)
- FastPfu DNA polymerase (TransGen Biotech, cat. no. AP221-03)
- High Pure dNTPs, 2.5 mM each (TransGen Biotech, cat. no. AD101-12)

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TABLE 1 | Primer sequences for sgRNA cloning and validation.

Step	Name	Sequence	Purpose
10	OsU3-F	5′-AAGGAATCTTTAAACATACGAACAGATC-3′	Colony PCR and sequencing primer for sgRNA vector verification
10	TaU6-F	5′-CCCAAGCTTGACCAAGCCCGTTATTCT-3′	Colony PCR and sequencing primer for sgRNA vector verification
1	Rice-Fwd	5′-GGCANNNNNNNNNNNNNNNNNNNN-3′	Cloning sgRNA into pOsU3-sgRNA
1	Rice-Rev	5′-AAACNNNNNNNNNNNNNNNNNNNN-3′	Cloning sgRNA into pOsU3-sgRNA Colony PCR primer for sgRNA vector verification
1	Wheat-Fwd	5′-CTTGNNNNNNNNNNNNNNNNNNNN-3′	Cloning sgRNA into pTaU6-sgRNA
1	Wheat-Rev	5′-AAACNNNNNNNNNNNNNNNNNNNN-3′	Cloning sgRNA into pTaU6-sgRNA Colony PCR primer for sgRNA vector verification

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- Chemically competent cells of *Escherichia coli* DH5á (TransGen Biotech, cat. no. CD201-02)
- TAE buffer, 10× (Cellgro, cat. no. 46-010-CM)
- Agarose (Invitrogen, cat. no. 16500500)
- Trans2K Plus II DNA marker (TransGen Biotech, cat. no. BM121-02)
- Ethidium bromide solution, 10 mg ml^{-1} (Sigma-Aldrich, cat. no. E8751)
- Plasmid miniprep kit (Axygen, cat. no. AP-MN-P-250)
- DNA gel extraction kit (Axygen, cat. no. AP-GX-250)
- Ampicillin, 100 mg ml^{-1}, sterile-filtered (Sigma-Aldrich, cat. no. A5354)
- Wizard Plus midipreps (Promega, cat. no. A7640)
- Glycerol (AMRESCO, cat. no. 0854)
- Annealing buffer, 10× (OriGene, cat. no. GE100007)

(Optional) ssDNA oligo template

- ssDNA oligo designed as described in Experimental design. Dilute the ssDNA to a final concentration of 100 µM with ddH2O. We recommend this higher oligo concentration, and we find that ssDNA oligos perform better than annealed oligos. The ssDNA oligo sequence used for the experiments shown in **Figure 5** is provided in **Supplementary Table 4**

Protoplast isolation and transformation

- 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.2 M, pH 5.7 (Sigma-Aldrich, cat. no. M8250); sterilize it with a 0.45-µm filter
- Mannitol, 0.8 M (Sigma-Aldrich, cat. no. M4125); sterilize it with a 0.45-µm filter
- $CaCl_2$, 1 M (Sigma-Aldrich, cat. no. C7902); sterilize it with a 0.45-µm filter
- KCl, 2 M (Sigma-Aldrich, cat. no. P3911); sterilize it with a 0.45-µm filter
- $MgCl_2$, 2 M (Sigma-Aldrich, cat. no. M9272); sterilize it with a 0.45-µm filter
- BSA (Sigma-Aldrich, cat. no. A6793)
- Cellulase R10 (Yakult Pharmaceutical Industry)
- Macerozyme R10 (Yakult Pharmaceutical Industry)
- PEG4000 (Sigma-Aldrich, cat. no. 95904)

PCR/RE and T7EI assay

- DNAquick plant system (Tiangen Biotech, cat. no. DP321-03)
- T7EI (ViewSolid Biotech, cat, no. E001L)
- T7EI buffer, 10× (ViewSolid Biotech, cat. no. B001)
- FastDigest PstI (Fermentas/Thermo Scientific, cat. no. FD0614)
- FastDigest SacI (Fermentas/Thermo Scientific, cat. no. FD1133)
- FastDigest EcoRV (Fermentas/Thermo Scientific, cat. no. FD0304)
- FastDigest MscI (Fermentas/Thermo Scientific, cat. no. FD1214)
- FastDigest buffer, 10× (Fermentas/Thermo Scientific, supplied with FastDigest restriction enzyme)
- EasyTaq DNA polymerase (TransGen Biotech, cat. no. AP111)
- pUC-T vector (CWBIO, cat. no. CW080)

Rice biolistic transformation

- Murashige and Skoog (MS) salt (Phytotech, cat. no. M519)
- N6B5 salt (Phytotech, cat. no. N492)
- Phytagel (Sigma-Aldrich, cat. no. P8169)
- Mannitol (AMRESCO, cat. no. 0122)
- Inositol (Sigma-Aldrich, cat. no. I7508)
- Sorbitol (AMRESCO, cat. no. 0691)
- Sucrose (Sigma-Aldrich, cat. no. S5390)
- Glutamine (Sigma-Aldrich, cat. no. G3126)
- Proline (Sigma-Aldrich, cat. no. P5607)
- 2,4-Dichlorophenoxyacetic acid (2, 4-D; Sigma-Aldrich, cat. no. D7299)
- 6-Benzylaminopurine (6-BA) (Sigma-Aldrich, cat. no. B3408)

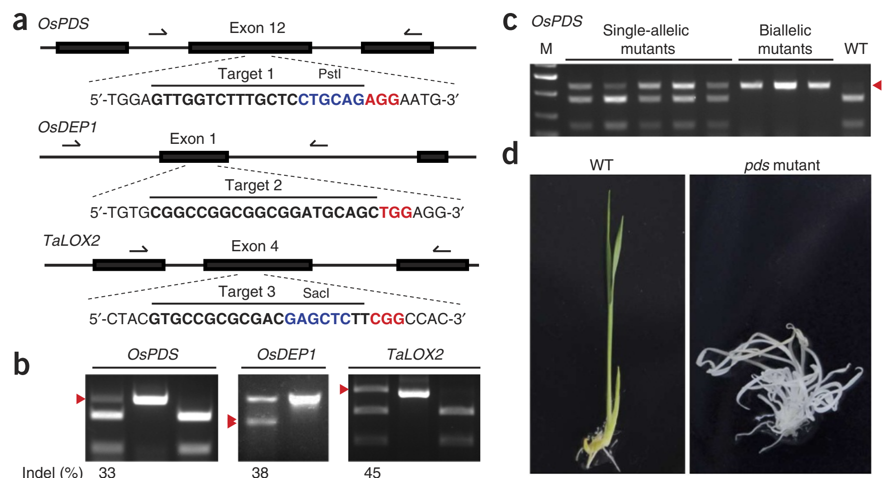

Figure 4 | Anticipated results for CRISPR/Cas-induced mutations. (**a**) Two of the three sgRNAs are designed to target the rice *PDS* and *DEP1* genes, respectively, and the remaining one is designed to target the wheat *LOX2* gene. Restriction enzyme sites are highlighted in blue letters; red letters indicate PAM sequences and black arrows indicate the primers used for PCR amplification. Boldface text indicates sgRNA targets. (**b**) Agarose gels illustrating mutations at three loci in transformed protoplasts. *OsPDS* and *TaLOX2* mutations are detected by the PCR/RE assay (red arrowheads indicate mutated bands), and those in the *OsDEP1* are detected by the T7EI assay (red triangles indicate cleaved mutated bands). (**c**) Agarose gel showing mutations in *OsPDS* in transgenic rice plants detected by the PCR/RE assay. Red triangle indicates mutated bands in single-allelic and biallelic mutants. (**d**) Phenotypes of the *pds* mutants. Biallelic mutants have the albino and dwarf phenotype. See **Supplementary Table 1** for sequence of sgRNA oligos, **Supplementary Table 2** for the calculated mutagenesis frequencies for each locus and **Supplementary Table 3** for sequence of PCR primers. WT, wild type.

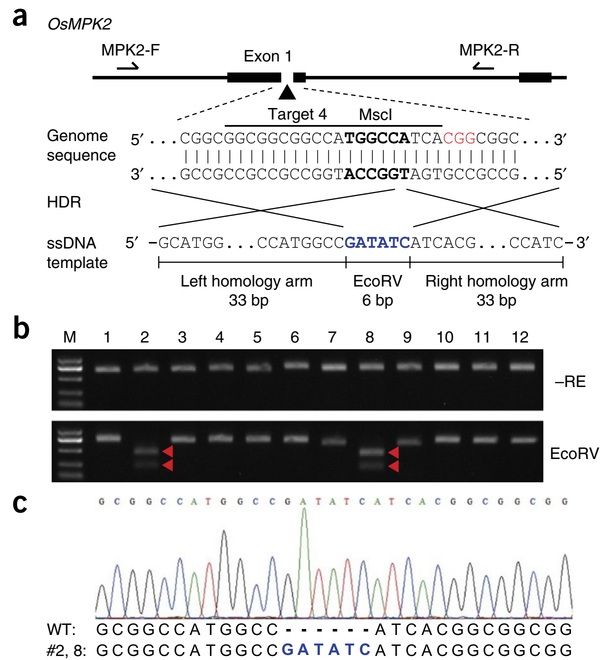

Figure 5 | Anticipated results for HDR-mediated genome modification in rice protoplasts. (**a**) Schematic of ssDNA template-mediated HDR in *OsMPK2*. The target 4 sequence is indicated and the PAM sequence is written in red. Boldface text in black indicates MscI site. The oligonucleotide donor (72 bp) is shown under the target site, with a 6-bp insertion (EcoRV site) labeled in blue; the full ssDNA oligo donor sequence can be found in **Supplementary Table 4**. (**b**) PCR amplification of protoplast genomic DNA predigested with MscI to enrich for CRISPR/Cas-induced mutations. Specific MPK2-F and MPK2-R primers are used. The enrichment PCR product was cloned into pEASY-Blunt vector. Lanes 1–12, representative PCR products of cloned alleles from digestion assays; –RE, PCR amplification of colonies with M13F/R primers; EcoRV, PCR products digested with EcoRV. Two cloned alleles (2 and 8, red arrowheads) with EcoRV insertions were identified. (**c**) The Sanger sequencing results for cloned allelles 2 and 8 show HDR-mediated targeting. Inserted sequences are labeled in blue.

- Hygromycin (Roche, cat. no. 10843555001)
- N-Z-Amine A (Sigma-Aldrich, cat. no. C7290)
- Kinetin (Sigma-Aldrich, cat. no. K0753)

EQUIPMENT

- PCR thermocycler (Bio-Rad)
- Environmentally controlled incubators (Eppendorf)
- Heating water bath (Changfeng)
- NanoDrop spectrophotometer (Thermo Scientific)
- Standard equipment and regents for agarose gel electrophoresis (Bio-Rad)
- Tissue culture plate, six wells (Evergreen, cat. no. 333-8028-01F)
- Tissue culture plate, 24 wells (Evergreen, cat. no. 333-8024-01F)
- Round-bottom centrifuge tubes, 50 ml (Haimeng)
- Standard microcentrifuge tubes, 1.5 ml (Eppendorf, cat. no. 0030 125.150)
- Single-edge razor blade (Feiying)
- Nylon mesh, 40 μm (BD Falcon)
- Syringe sterilization filter, 0.45 μm (Sartorius Stedim Biotech, cat. no. 21423103)
- Fluorescence microscope (Olympus)
- PDS1000/He particle bombardment system (Bio-Rad)
- Digital gel imaging system (BioDoc-It, UVP, cat. no. 97-0256-02)
- Gel quantification software (ImageJ from the NIH, USA, available at http://rsbweb.nih.gov/ij/)

REAGENT SETUP

Rice plants for protoplast isolation Use the rice cultivar Nipponbare for protoplast isolation, as its genome has been sequenced. Other cultivars may also work using this protocol. Sterilize dehulled seeds with 75% (vol/vol) ethanol followed by washing them for 1 min, draining them and then incubating the seeds in 2.5% (wt/vol) sodium hypochlorite for 20 min; finally, wash the seeds 5–7 times with sterile water. Pour ~60 ml 1/2 MS solid medium into a round glass cup (diameter 66 mm and height 180 mm). Plate 10–15 sterilized seeds onto the medium and cover the cup with sterilized plastic film. Grow the seeds at 28°C with a photoperiod of 16 h of light (~150 μmol m^{-2} s^{-1}) and 8 h of dark for ~14–20 d.

Wheat plants for protoplast isolation We use the common wheat cultivar Kenong 199 for protoplast isolation; other cultivars may also work using this protocol. Grow the seeds at 25 °C with a photoperiod of 16 h of light and 8 h of dark, as described above for rice plants, for ~7–14 d.

Cell wall–dissolving enzyme solution Prepare 20 mM MES (pH 5.7) containing 1.5% (wt/vol) cellulase R10, 0.75% (wt/vol) macerozyme R10, 0.6 M mannitol and 10 mM KCl. Warm the solution at 55 °C for 10 min to inactivate DNases and proteases and to enhance enzyme solubility. Cool the solution to room temperature (RT; 22–25 °C) and add 10 mM $CaCl_2$ and 0.1% (wt/vol) BSA. Finally, filter-sterilize the enzyme solution. The enzyme solution should be freshly prepared.

W5 solution Prepare 2 mM MES (pH 5.7) containing 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl. The prepared solution can be stored at RT for up to 2 months.

Washing and incubation (WI) solution Prepare 4 mM MES (pH 5.7) containing 0.5 M mannitol and 20 mM KCl. The prepared solution can be stored at RT for up to 2 months.

Mannitol $MgCl_2$ (MMG) solution Prepare 4 mM MES (pH 5.7) containing 0.4 M mannitol and 15 mM $MgCl_2$. The prepared MMG solution can be stored at RT for up to 2 months.

PEG solution Prepare 40% (wt/vol) PEG4000 in ddH_2O containing 0.2 M mannitol and 100 mM $CaCl_2$. ▲ **CRITICAL** The PEG solution should be freshly prepared but at least 1 h before transformation to completely dissolve the PEG.

1/2 MS solid medium Add 2.215 g of MS salt, 30 g of sucrose and 0.1 g of inositol to 700 ml of distilled water, and make up the volume to 1,000 ml. Adjust the pH to 5.8 and add 3 g of phytagel. Autoclave the medium at 121 °C for 20 min. Pour ~55 ml of medium into a round glass cup. Store it at 4 °C for up to 6 weeks.

Osmotic medium Add 4.43 g of MS salt, 5 ml of 1 mg ml^{-1} 2,4-D, 90 g of mannitol and 30 g of sucrose to 700 ml of distilled water, and make up the volume to 1,000 ml. Adjust the pH to 6.0 and add 3.5 g of phytagel. Autoclave the medium at 121 °C for 20 min. Pour ~25 ml of medium in sterile Petri dishes. Store the Petri dishes at 4 °C for up to 6 weeks in a dark container to protect them from light.

Recovery medium Add 4.1 g of N6B5 salt, 2 ml of 1 mg ml^{-1} 2,4-D, 0.5 g of glutamine, 0.1 g of inositol, 2.8 g of proline, 0.5 g of N-Z-Amine A and 30 g of sucrose to 700 ml of distilled water and make up the volume to 1,000 ml. Adjust the pH to 5.8 and add 4 g of phytagel. Autoclave the medium at 121 °C for 20 min. Pour ~25 ml of medium in sterile Petri dishes. Store the dishes at 4 °C for up to 6 weeks in a dark container.

Selection medium Add 4.1 g of N6B5 salt, 2 ml of 1 mg ml^{-1} 2,4-D, 0.5 g of glutamine, 0.1 g of inositol, 2.8 g of proline, 0.5 g of N-Z-Amine A and 30 g of sucrose to 700 ml of distilled water, and make up the volume to 1,000 ml. Adjust the pH to 5.72 and add 4 g of phytagel. Autoclave the medium at 121 °C for 20 min. Cool it to 50 °C and add 1 ml of 50 mg ml^{-1} hygromycin. Pour ~25 ml of medium in sterile Petri dishes. Store the dishes at 4 °C for up to 6 weeks in a dark container.

Regeneration medium Add 4.1 g of N6B5 salt, 1 ml of 2 mg ml^{-1} 6-BA, 0.2 ml of 1 mg ml^{-1} kinetin, 0.1 g of inositol, 20 g of sorbitol, 0.5 g of N-Z-Amine A and 30 g of sucrose to 700 ml of distilled water, and make up the volume to 1,000 ml. Adjust the pH to 5.72 and add 4.6 g of phytagel. Autoclave the medium at 121 °C for 20 min. Cool it to 50 °C and add 0.6 ml of 50 mg ml^{-1} hygromycin. Pour ~25 ml of medium in sterile Petri dishes. Store the dishes at 4 °C for up to 6 weeks in a dark container.

Rooting medium Add 4.43 g of MS salt, 0.1 g of inositol and 30 g of sucrose to 700 ml of distilled water, and make up the volume to 1,000 ml. Adjust the pH to 5.8 and add 2.4 g of phytagel. Autoclave the medium at 121 °C for 20 min. Cool it to 50 °C, and add 0.6 ml of 50 mg ml^{-1} hygromycin. Pour ~25 ml of medium in sterile Petri dishes. Store the dishes at 4 °C for up to 6 weeks in a dark container.

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PROCEDURE

Cloning target-specific sgRNA oligos into sgRNA scaffold vectors for transient expression in protoplasts ● TIMING 4 d

1| Dilute each forward and reverse target-specific sgRNA oligos to a final concentration of 10 μM. Prepare the following mixture to anneal the sgRNA oligos:

Component	Amount (μl)	Final
Annealing buffer, 10×	2	1×
Rice-Fwd or Wheat-Fwd (10 μM)	9	4.5 μM
Rice-Rev or Wheat-Rev (10 μM)	9	4.5 μM
Total	20	

2| Anneal the oligos in a thermocycler using the following conditions:

Cycle number	Condition
1	95 °C, 5 min
2	95–25 °C, –1 °C min^{-1}, 70 min
3	10 °C, Hold

3| Digest the appropriate scaffold plasmid (pOsU3-sgRNA for rice, pTaU6-sgRNA for wheat) with AarI in a 50-μl reaction mixture, as tabulated below:

Component	Amount (μl)	Final
AarI buffer (supplied with AarI), 10×	5	1×
Oligonucletide (supplied with AarI), 50×	1	1×
AarI, 2 U μl^{-1}	2	4 units
pOsU3-sgRNA or pTaU6-sgRNA	Variable	2 μg
ddH_2O	To 50	—

4| Incubate the digestion reaction at 37 °C for 3–16 h.
▲ **CRITICAL STEP** A longer incubation time is better.

5| Run the digested product on a 1.2% (wt/vol) agarose gel in TAE buffer using standard protocols. A successful digestion reaction should yield a single 3.2-kb band for pOsU3-sgRNA or a single 2.9-kb band for pTaU6-sgRNA.

6| Cut out the band under UV light and purify the vector DNA with a gel purification kit.
■ **PAUSE POINT** The purified vector DNA can be stored at –20 °C for several months.

7| Ligate the annealed sgRNA oligos (from Step 2) into the digested vector. We recommend also setting up a no-insert-vector-only negative control for ligation. Set up the following reaction and incubate it at 22 °C for 1 h:

Component	Amount (μl)	Final
T4 ligation buffer, 10×	1	1×
Digested vector (Step 6)	Variable	50 ng
Annealed sgRNA oligos (Step 2)	3	
T4 ligase, 5 U μl^{-1}	0.5	2.5 units
ddH_2O	To 10	

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8| Transform the ligation reaction into competent *E. coli* cells according to the manufacturer's instructions, and plate the cells onto an LB plate containing ampicillin. Incubate the plate at 37 °C overnight.
? TROUBLESHOOTING

9| Isolate plasmid DNA from 5-ml cultures grown from individual colonies using a plasmid miniprep kit according to the manufacturer's instructions. Two or three colonies are sufficient.

10| Sequence the plasmids with primer OsU3-F (for pOsU3-sgRNA) or TaU6-F (for pTaU6-sgRNA; **Table 1**) to verify that the clones harbor the correct sgRNA.
? TROUBLESHOOTING

11| Isolate plasmid DNA from 100-ml cultures of correct clones using a midiprep kit according to the manufacturer's instructions. The final concentration should be at least 1 µg μl^{-1} in a total volume of 250 µl with an $A_{260/280}$ ratio range of 1.7–1.9.
■ **PAUSE POINT** Midiprep plasmid DNA can be stored at –20 °C for several months.

Isolation of protoplasts ● TIMING 1 d
▲ **CRITICAL** The quality and quantity of protoplasts is important for successful transient protoplast transformation.
▲ **CRITICAL** The protocol for rice protoplast isolation and transformation is based on previous work[54] with some modifications.

12| Isolate fresh protoplasts from rice sheaths, as described in option A, or from wheat leaves, as described in option B. All steps are performed at RT.
(A) Isolation of rice protoplasts ● TIMING 1 d
(i) Bundle together the stems and sheaths of ~30 rice seedlings. Cut them into latitudinal strips (by transecting) of ~0.5 mm in width using very sharp razor blades. Usually 1×10^7 protoplasts can be isolated from 100–120 seedlings, and they can be used in 20 separate transformations with 5×10^5 cells per transformation.
(ii) Transfer the strips into a Petri dish with 0.6 M mannitol, and incubate them for 10 min in the dark for quick plasmolysis.
(iii) Filter through nylon meshes, transfer the strips into a 150-ml conical flask containing 50 ml of filter-sterilized enzyme solution and wrap the flask with aluminum foil.
(iv) Vacuum-infiltrate the strips with cell wall–dissolving enzymes by applying a vacuum (~380–508 mmHg) for 30 min in the dark. Next, incubate the strips in the dark for 5–6 h with gentle shaking (60–80 r.p.m.) at RT.
▲ **CRITICAL STEP** Infiltration under vacuum pump is essential for a good protoplast yield.
(v) After enzymatic digestion, add 50 ml of W5 solution to the conical flask, and then shake it gently by hand for 10 s to release the protoplasts.
(vi) Collect the protoplasts into three or four 50-ml round-bottomed centrifuge tubes after filtering the mixture through 40-µm nylon meshes and washing the strips on the surface of the nylon mesh 3–5 times with W5 solution.
▲ **CRITICAL STEP** Washing the strips 3–5 times with W5 solution is essential for a high protoplast yield.
(vii) Centrifuge at 250*g* for 3 min at room temperature (RT) in a swinging bucket rotor and remove the supernatant by pipetting. Re-suspend the protoplasts in 10 ml of W5 solution and collect them into a 50-ml round-bottomed tube.
▲ **CRITICAL STEP** Protoplasts are fragile, so gentle manipulation is necessary. The centrifuge speed should not be too high; we recommend a speed of 250*g*.
(viii) Centrifuge the protoplasts at 250*g* for 3 min at RT, remove the supernatant by pipetting and resuspend the protoplasts in 4 ml of MMG solution at a final concentration of 2.5×10^6 cells per ml.
(ix) (Optional) Determine the protoplast concentration under a microscope (×100) with a hemocytometer.
? TROUBLESHOOTING
(B) Isolation of wheat protoplasts ● TIMING 1 d
(i) Bundle and cut healthy fresh wheat leaves into latitudinal 0.5-mm strips with a sharp razor blade. Proceed as described in Step 12A(ii–vi).
▲ **CRITICAL STEP** Usually 20–30 seedlings can generate 1×10^7 protoplasts, which can be transformed with 20 plasmid samples (5×10^5 cells per sample).

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(ii) Centrifuge the protoplasts at 80*g* for 3 min at RT in a swinging bucket rotor, and remove the supernatant by pipetting. Resuspend the protoplasts in W5 solution and keep them on ice for 30 min.
▲ **CRITICAL STEP** As wheat protoplasts are very fragile, we usually centrifuge them at 80*g*.
▲ **CRITICAL STEP** Wheat protoplasts should be kept on ice for 30 min for good transformation efficiency.

(iii) Intact protoplasts should have settled to the bottom of the tube by gravity after 30 min; remove as much supernatant as possible by pipetting without touching the protoplast pellet, and then resuspend the pellet in 4 ml of MMG solution at a final concentration of 2.5×10^6 cells per ml.
▲ **CRITICAL STEP** Remove the supernatant directly without centrifugation using a pipette.

(iv) (Optional) Determine the protoplast concentration under the microscope (×100) with a hemocytometer.
? TROUBLESHOOTING

PEG-mediated transformation of protoplasts and extraction of genomic DNA ● TIMING 2–3 h hands-on time; 2 d total time
▲ **CRITICAL** The following manipulations are performed at RT.
▲ **CRITICAL** Always set up a transformation with a GFP reporter plasmid as a positive control.

13| Prepare a 40-μl transformation mix in a 2-ml microcentrifuge tube. For rice transformation, add 20 μg of pOsU3-sgRNA and 20 μg of pJIT163-2NLSCas9 (from Step 16). For wheat transformation, add 20 μg of pTaU6-sgRNA and 20 μg of pJIT163-2NLSCas9 (from Step 16). For HDR-mediated gene targeting in rice or wheat, also include 10 μl (100 μM) of ssDNA oligo.

14| Add 200 μl of protoplasts (5×10^5 cells) and mix them gently by tapping the tube.

15| Add 220 μl of freshly prepared PEG solution and mix thoroughly by gently tapping the tube.

16| Incubate the mixture for 15–20 min in the dark. Add 880 μl of W5 solution to the tube and mix it well by inverting the tube to stop the transformation process.

17| For rice protoplasts, centrifuge them at 250*g* for 3 min at RT. For wheat protoplasts, centrifuge them at 80*g* for 3 min at RT. Remove the supernatant.

18| Resuspend the protoplasts gently; rice protoplasts should be resuspended in 2 ml of WI solution, and wheat protoplasts should be resuspended in 2 ml of W5 solution.

19| Transfer the protoplasts into six-well plates (12-well or 24-well plates can also be used for larger-scale experiments).

20| Wrap the plates in aluminum foil and incubate them at 23 °C for at least 48 h.
▲ **CRITICAL STEP** A longer incubation time is better, but do not exceed 72 h.

21| Check the condition of the protoplasts under a microscope; the healthy cells should appear full and round. Check the transformation efficiency by counting the number of GFP-fluorescing cells in the positive control using a fluorescence microscope. In general, there should be ~70–80% GFP-positive cells for both rice and wheat.
? TROUBLESHOOTING

22| Resuspend protoplasts by pipetting, and transfer them into 2-ml microcentrifuge tubes.

23| Collect the protoplasts by centrifuging at 12,000*g* for 2 min at RT; remove the supernatant.

24| Extract genomic DNA with the DNAquick plant system according to the manufacturer's instructions.

25| Determine the DNA concentration with a NanoDrop spectrophotometer; the usual range is 30–60 ng μl^{-1} in a total volume of 30 μl.
■ **PAUSE POINT** Extracted genomic DNA can be stored at –20 °C for several months.

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Detection of mutations in protoplasts ● TIMING ~3 d

26| Set up the following PCR to amplify the genomic region targeted for mutagenesis. If HDR-mediated mutagenesis was performed (at Step 13), the genomic DNA can be enriched by restriction enzyme digestion before PCR, as described in **Box 2**.

▲ CRITICAL STEP If the PCR/RE assay is to be used to detect mutations (Step 29A), make sure that the restriction enzyme site in the sgRNA target is the only one in the amplicon.

▲ CRITICAL STEP Before detecting the mutations, we recommend that each primer pair used for PCR amplification be tested with wild-type control samples.

▲ CRITICAL STEP It is important to use a high-fidelity polymerase to reduce the error rate of the PCR amplification. In addition, wild-type samples should be used as negative controls.

Component	Amount (μl)	Final
T4 ligation buffer, 10×	1	1×
Digested vector (Step 6)	Variable	50 ng
Annealed sgRNA oligos (Step 2)	3	
T4 ligase, 5 U μl^{-1}	0.5	2.5 units
ddH_2O	To 10	

Component	Amount (μl)	Final concentration
Fast Pfu buffer, 5×	10	1×
dNTP, 10 mM (2.5 mM each)	5	1 mM
PCR-Fwd primer, 10 μM	2	0.4 μM
PCR-Rev primer, 10 μM	2	0.4 μM
Fast Pfu polymerase, 2.5 U μl^{-1}	1	2.5 units
DNA template (undigested or digested)	5	3–6 ng μl^{-1}
ddH_2O	To 50	

27| Perform a PCR with the following parameters; 35 amplification cycles are enough.

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–36	95 °C, 20 s	60 °C, 20 s	72 °C, 15 s
37			72 °C, 5 min

28| Run 5 μl of the PCR products on a 1.2% (wt/vol) agarose gel in TAE buffer to check for the desired fragments, and then estimate the yield of products from the band intensities.

? TROUBLESHOOTING

29| To detect mutations generated by NHEJ, use the PCR/RE assay (option A), or the T7EI assay (option B). To detect precise HDR-mediated genome modifications, follow option C. Option A (PCR/RE) can only be used if an appropriate restriction enzyme site is located just upstream of the PAM sequence; the T7E1 assay should be used when no appropriate restriction enzyme site exists.

(A) PCR/RE assay ● TIMING **3 d**

(i) Use the PCR product (from Step 27) to set up the following restriction enzyme digestion reaction:

Component	Amount (μl)	Final
FastDigest Green buffer, 10×	5	1×
Enzyme	2	
PCR product (Step 27)	8	
ddH_2O	To 50	

▲ CRITICAL STEP Digest the PCR product from a wild-type sample as a negative control.

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(ii) Mix the reaction well and spin it down briefly. Incubate the reaction at 37 °C for 1 h.
(iii) Run all 50-µl digestion products on a 2% (wt/vol) agarose gel in TAE buffer using standard protocols. Include a DNA ladder and negative control digests on the same gel. An uncleaved band only in the CRISPR/Cas-treated sample, and not in the negative control, is indicative of an indel mutation.
? TROUBLESHOOTING
(iv) Estimate the indel frequency. Measure the intensity of the PCR amplicon and cleaved bands with ImageJ or other gel quantification software. Calculate the indel frequency from the following formula:

$$\text{indel}(\%) = 100 \times a \,/\, (a + b + c)$$

where a is the intensity of the undigested PCR product, and b and c are the intensities of the two digested products. A sample gel is shown in **Figure 4a,b**.
(v) Purify the undigested band with a gel purification kit according to the manufacturer's instructions and identify the mutations by cloning and sequencing of plasmid DNA isolated from overnight cultures of single colonies.
(vi) If the indel frequency is >10%, the sgRNA can be used for stable transformation of plants. To stably transform rice, proceed to Step 30.

(B) T7EI assay ● TIMING 3 d
(i) By using the PCR product from Step 27, set up the following annealing reaction to form DNA heteroduplexes:

Component	Amount (µl)
T7EI buffer, 10×	1.1
PCR product (Step 27)	5
ddH_2O	4.4
Total	10.5

▲ **CRITICAL STEP** The reaction can be scaled up if necessary.
(ii) Run the annealing reaction in a thermocycler using the following conditions: 95 °C for 5 min, and then ramp it down to 15 °C (–10 °C/min).
(iii) Use the annealed heteroduplex (Step 29B(ii)) to set up the following T7EI nuclease digestion:

Component	Amount (µl)	Final
Annealed heteroduplex (Step 29B(ii))	10.5	
T7EI, 5 U μl^{-1}	0.5	2.5 units
Total	11	

(iv) Mix well and spin the mixture down briefly. Incubate the reaction at 37 °C for 1 h.
(v) Run all 11-µl digestion products on a 2% (wt/vol) agarose gel in TAE buffer using standard protocols. Include a DNA ladder and negative controls on the same gel. If the digested bands are only observed in the CRISPR/Cas-treated sample, and not in the negative control, an indel mutation has been isolated.
? TROUBLESHOOTING
(vi) Estimate the indel frequency. Measure the intensity of the PCR amplicon band and digested bands using ImageJ or other gel quantification software. Calculate the indel frequency from the following formula:

$$\text{indel}(\%) = 100 \times \left(1 - \sqrt{1 - \frac{b+c}{a+b+c}}\right)$$

where a is the intensity of the undigested PCR product, and b and c are the intensities of the two digested products. A sample gel is shown in **Figure 4b**.
(vii) Identify the mutations by cloning PCR products (from Step 27) into pEASY-Blunt cloning vector and sequencing plasmid DNA isolated from overnight cultures of single colonies.
(viii) If the indel frequency is >10%, the sgRNA can be used for stable transformation of plants. To stably transform rice, proceed to Step 30.

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(C) Assessment of HDR-mediated modification by subcloning and sequencing ● TIMING 3 d

(i) Purify the PCR products from the gel (Step 27) using a DNA gel extraction kit.

■ **PAUSE POINT** The purified PCR products can be stored at −20 °C for several months.

(ii) Ligate and clone the PCR products into pEASY-Blunt cloning vector, and then transform *E. coli*-competent cells according to the manufacturer's instructions.

(iii) Assuming that an appropriate restriction enzyme site exists, set up the following colony PCR on random single colonies to detect the modification by the PCR/RE assay:

Component	Amount (μl)	Final concentration
EASY Taq buffer, 10×	2.5	1×
dNTP, 10 mM (2.5 mM each)	2.5	1 mM
M13F, 10 μM	1	0.4 μM
M13R, 10 μM	1	0.4 μM
Easy Taq polymerase, 5 U μl^{-1}	0.2	1 unit
Colony (Step 29C(ii))		
ddH_2O	To 25	

(iv) Use the following PCR conditions; 30 amplification cycles are sufficient.

Cycle number	Denature	Anneal	Extend
1	98 °C, 3 min		
2–31	98 °C, 30 s	60 °C, 30 s	72 °C, 30 s
32			72 °C, 5 min

(v) After running the PCR, run 5 μl of product on a 1.2% (wt/vol) agarose gel in TAE buffer to verify that the PCR has been successful.

(vi) Digest the colony PCR products with the appropriate restriction enzyme at 37 °C for 1 h, as tabulated below. In the example provided in **Figure 5a**, an EcoRV site is inserted into the mitogen-activated protein kinase 2 (*MPK2*) gene by HDR, so the PCR product is digested with EcoRV.

Component	Amount (μl)	Final
FastDigest Green buffer, 10×	2	1×
Restriction enzyme (e.g., EcoRV)	1	
PCR product (Step 29C(iv))	5	
ddH_2O	To 20	

(vii) Run all the digested products on a 1.2% (wt/vol) agarose gel in TAE buffer (**Fig. 5b**).

(viii) Sequence the undigested colony PCR products (Step 29C(iv)) with appropriate sequencing primers (MPK2-F or MPK2-R) to identify clones with the correct insertion (**Fig. 5c**).

(ix) Estimate HDR efficiency (%) using the following formula: 100 × a/b, where a is the number of colonies containing the correct oligo insertion, and b is the number of colonies tested.

▲ **CRITICAL STEP** If an enrichment process (**Box 2**) is carried out, then the HDR efficiency should not be calculated by the formula (100 × a/b). The true efficiency is much lower.

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Stable transformation of rice using the biolistic method ● TIMING 13–17 weeks

30| Place six- to nine-week-old embryogenic calli (30–40 pieces) of the rice cultivar Nipponbare on an osmotic medium for 4 h before bombardment.

31| Set up the following mix so that plasmid DNAs are in a 1:1:1 molar ratio before bombardment:

Component	Amount (μl)
Gold particles (diameter 0.6 μm), 40 mg ml^{-1}	120
pOsU3-sgRNA, 1 μg $μl^{-1}$	5
pJIT163-2NLSCas9, 1 μg $μl^{-1}$	5
pAct1-HPT, 1 μg $μl^{-1}$	5
Spermidine, 0.1 M	40
$CaCl_2$, 2.5 M	100

32| Perform biolistic transformation using a PDS1000/He particle bombardment system with a target distance of 6.0 cm from the stopping plate at a helium pressure of 1,100 p.s.i.

33| Incubate bombarded calli on osmotic medium at 25–26 °C in the dark overnight.

34| Transfer the calli to recovery medium without selection and incubate them at 28 °C in the dark for 7 d.

35| Transfer the calli to selection medium and incubate them at 28 °C in the dark for 5–6 weeks.

36| Transfer hygromycin-resistant calli to regeneration medium and incubate them at 28 °C with a photoperiod of 16 h of light (~150 μmol m^{-2} s^{-1}) and 8 h of dark for 4 weeks.

37| Transfer the plantlets with roots to rooting medium in containers and allow them to develop further.

Detection and sequencing of mutations in transgenic rice ● TIMING 3 d

38| Extract genomic DNA from leaves of transgenic rice plants using the DNAquick plant system according to the manufacturer's instructions. Adjust the extracted DNA to a final concentration of 100–200 ng $μl^{-1}$ with ddH_2O.

39| PCR-amplify the fragments containing the sgRNA target region, and run an agarose gel as described in Steps 26–28.

40| Detect indel mutations in the PCR products either by the PCR/RE assay (Step 29A(i–iii)) or by the T7EI assay (Step 29B).

▲ **CRITICAL STEP** For PCR/RE (Step 29A), we do not estimate indel frequency from band intensities as described in Step 29A(iv). After restriction enzyme digestion, there are three possibilities for rice. If the PCR products can be digested completely, then there are no mutations; if they are only partially digested, then they carry single-allelic mutations; if there are no digested products, then they carry biallelic mutations (**Fig. 4c**).

▲ **CRITICAL STEP** The T7EI assay (Step 29B) cannot detect homozygous biallelic mutants because no mismatch arises after DNA heteroduplex formation. Sequencing the PCR products in Step 39 may be an alternative.

? TROUBLESHOOTING

41| Verify the sequence of each mutation by sequencing PCR products (from Step 39) to determine the precise nature of each mutation.

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? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
8	Colonies growing on negative control plate	Incomplete digestion of pOsU3-sgRNA plasmid	Increase the amount of enzyme or increase digestion time
10	Wrong sgRNA sequences	Incomplete digestion of sgRNA plasmids or ligation failure	Sequence more colonies; redigest pOsU3-sgRNA plasmid; reanneal sgRNA oligos
12A(ix), 12B(iv)	Low protoplast yield or poor protoplast quality	Poor seedling condition or inefficient enzyme digestion	Check seedlings' age, development and morphology; check activity of enzyme solution (enzyme quality)
21	Low transformation efficiency	Low-quality DNA, PEG solution problem or poor protoplast quality	We use a Promega midiprep plasmid kit for DNA purification; PEG quality depends on the source and we use freshly prepared solution; re-isolate protoplasts
28	No amplification or multiple bands	Incorrect PCR conditions or low template concentration; nonspecific primers	Improve PCR condition; increase template amounts; redesign primers; change DNA polymerase
29A(iii), 29B(v)	Nonspecific or smeared bands on gel; no uncleaved band in the PCR/RE assay or no cleaved bands in the T7EI assay	Overdigestion by RE or T7EI; improper template annealing; input PCR templates too low; sgRNA for the locus does not mediate cleavage	Shorten digestion time to <1 h; reanneal templates; increase input PCR templates; design additional sgRNA
40	No or limited numbers of mutant plants	Inefficient sgRNA-mediated cleavage; small transgenic population; detection assay missed mutants	Transform a larger population; re-design a more efficient sgRNA; do a positive control for the PCR/RE or T7EI assay

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● TIMING

Steps 1–11, cloning target-specific sgRNA oligos into sgRNA scaffold vectors for transient expression in protoplasts: 4 d
Step 12, isolation of protoplasts: 1 d
Steps 13–25, PEG-mediated transformation of protoplasts and extraction of genomic DNAs: 2–3 h hands-on time; 2 d total time
Steps 26–29, detection of mutations in protoplasts: ~3 d
Steps 30–37, stable transformation of rice using the biolistic method: 13–17 weeks
Steps 38–41, detection and sequencing of mutations in transgenic rice: 3 d
Box 2, Enrichment of genomic DNA by restriction enzyme digestion: 1 h

ANTICIPATED RESULTS

This protocol generates sgRNA expression vectors that can be used to target any gene of interest in rice and wheat. It provides a quick and effective method for testing sgRNA activity *in vivo* by transient expression in protoplasts. In our experience, seven or eight sgRNAs out of ten induce >20% indels (**Fig. 4b**) in rice. For wheat, the proportion is about three or four sgRNAs out of ten. The reason for the lower indel yield in wheat may be the complex genomic background. We summarize several key elements of sgRNA design and mutation identification in wheat in **Box 1.** In rice protoplasts, we induce HDR using an ssDNA template together with sgRNA and Cas9 (**Fig. 5**). By stable biolistic transformation of sgRNA and Cas9, we can obtain rice mutants with single-allelic and biallelic mutations in the T_0 generation (**Fig. 4c**), and rice biallelic *pds* mutants have the expected phenotype (**Fig. 4d**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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