Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 *in vitro* transcripts or ribonucleoproteins

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In recent years, CRISPR/Cas9 has emerged as a powerful tool for improving crop traits. Conventional plant genome editing mainly relies on plasmid-carrying cassettes delivered by *Agrobacterium* or particle bombardment. Here, we describe DNA-free editing of bread wheat by delivering *in vitro* transcripts (IVTs) or ribonucleoprotein complexes (RNPs) of CRISPR/Cas9 by particle bombardment. This protocol serves as an extension of our previously published protocol on genome editing in bread wheat using CRISPR/Cas9 plasmids delivered by particle bombardment. The methods we describe not only eliminate random integration of CRISPR/Cas9 into genomic DNA, but also reduce off-target effects. In this protocol extension article, we present detailed protocols for preparation of IVTs and RNPs; validation by PCR/restriction enzyme (RE) and next-generation sequencing; delivery by biolistics; and recovery of mutants and identification of mutants by pooling methods and Sanger sequencing. To use these protocols, researchers should have basic skills and experience in molecular biology and biolistic transformation. By using these protocols, plants edited without the use of any foreign DNA can be generated and identified within 9–11 weeks.

INTRODUCTION

Bread wheat, an allohexaploid species (*Triticum aestivum* $2n = 6x = 42$, AABBDD), is a globally cultivated food crop. To fulfill the increasing demand for food to feed the expanding population, a rapid, precise and safe tool for breeding by recombinant methods is needed. However, introducing targeted mutations in wheat is difficult because of its complex genetic structure, giant genome (17 Gb) and recalcitrance to transformation[1]. Genome editing, based on changes induced by sequence-specific nucleases, has been used to generate precise genome modifications across a broad range of plants, including wheat[2–4]. Recently, the CRISPR (clustered regularly interspaced short palindromic repeats)/associated nuclease Cas9 (CRISPR/Cas9) system has largely replaced its predecessors, zinc-finger nucleases and transcription activator-like effector nucleases, due to its unprecedented simplicity, efficiency and versatility[5–9]. Over the past 4 years, the CRISPR/Cas9 system has been widely adopted in wheat[10], rice[11,12], maize[13–15], soybean[16,17], potato[18], tomato[19] and many other crops[20–22], mainly for generating mutants. CRISPR/Cas9 components can be delivered to cells in three forms, DNA expression plasmids (DNA), IVTs and preassembled RNPs[23], and by using several different methods. In most of the work to date, mutant plants have been produced by introducing CRISPR/Cas9 plasmids into plant cells by *Agrobacterium tumefaciens*–mediated T-DNA transfer or biolistic bombardment[24]. Under these conditions, CRISPR/ Cas9 DNA is often integrated into unknown genomic sites, which can enhance off-target cleavage as a result of constitutive expression of Cas9 and sgRNA. Furthermore, small fragments of the CRISPR/Cas9 constructs can act as filler DNA inserted into on-target and/or off-target sites[25,26]. These possibilities of foreign DNA integration and off-target effects have limited the adoption of these methods in crop breeding. The use of CRISPR/Cas9 IVTs and RNPs has been shown to avoid the random integration of foreign DNA and to reduce the frequency of off-target effects in many species: mouse[27,28], *Caenorhabditis elegans*[29] and zebrafish[30], as well as a number of cell lines[31–34]. Recently, Woo *et al.*[35] obtained genome-modified lettuces by delivering CRISPR/Cas9 RNPs into protoplasts by PEG-mediated transfection. Similar mutations have been reported in protoplasts of *Arabidopsis*[35], tobacco[35], rice[35], petunia[36], and grapevine and apple[37], but no mutant plants were regenerated. For most plants, particularly for major monocot food crops such as wheat, regenerating plants from protoplasts is still a challenge. Simple, specific and efficient DNA-free genome editing methods for wheat are greatly needed.

Development of the protocol

The protocol described here is an extension of our previous method for genome editing with CRISPR/Cas9 DNA constructs in rice and wheat[38]. In this protocol, we combine reliable methods for delivering CRISPR/Cas9 IVTs[39] or RNPs[40] by particle bombardment with selection-free tissue culture methods and mixed-pool screening approaches. Using particle bombardment, we deliver mixtures of *in vitro*-transcribed Cas9 mRNA and sgRNA (IVTs), or pre-assembled Cas9-sgRNA RNPs, into wheat embryos. The CRISPR/Cas9 is transiently expressed (in the case of IVTs) and functional, and is then degraded, after which the bombarded cells are allowed to develop into calli and then into regenerated plants without selection, and mutants are identified by mixed pool screening.

These methods proved successful in two wheat varieties, Kenong199 (ref. 41) and YZ814 (H. Zhang, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, personal communication). We found that RNP-mediated editing, in particular, not only eliminated foreign DNA integration, but also reduced off-target effects more efficiently than using CRISPR/Cas9 DNA[40]. Moreover, we made the transformation protocol in wheat simpler

than that in conventional genome editing based on *Agrobacterium* or biolistic transformation by eliminating the lengthy, costly and labor-intensive antibiotic selection step. In the selection-free culture method, there is no need to optimize the type and concentration of antibiotic for each different genotype, so the plant regeneration procedure is simplified and can more easily be applied to different genotypes. Furthermore, to overcome the obstacle of genotyping hundreds of seedlings under selection-free conditions, we developed a mutant screening method using mixed pools of three to four seedlings, usually generated from a single embryo. In summary, we have combined a delivery method, a selection-free regeneration method and a genotyping procedure to create an effective DNA-free editing method for wheat.

Overview of the procedure

In this protocol, we describe a method for DNA-free genome editing in wheat with CRISPR/Cas9 IVTs or ribonucleoproteins. sgRNA cloning and evaluation is performed following our previous CRISPR/Cas9 DNA editing protocol[38]. The entire workflow is summarized in **Figure 1** and is divided into four major stages: preparation of IVTs or RNPs (Steps 1–14), validation of RNPs (Steps 15–30), delivery of IVTs or RNPs (Steps 31–46), and mutant recovery and identification (Steps 47–61).

Advantages and limitations

The main advantages of this protocol are delineated in the following paragraphs.

Elimination of transgene integration. Both the CRISPR/Cas9 IVTs and RNPs are based on a DNA-free and selection-free strategy. These reagents can function without being integrated into the host plant genome. Hence, the edited plants generated should be more acceptable to the public.

Low off-target effects. Because CRISPR/Cas9 DNA can be integrated into the host genome and continuously expressed, this can extend its expression time and expression level, as well as lead to continuous cleavage of genomic DNA at on- or off-target sites. By contrast, Cas9 IVTs are only transiently expressed, and defined amounts of Cas9 RNPs can be delivered into plants cells, where they are rapidly degraded. Compared with the conventional CRISPR/Cas9 DNA-based method, using CRISPR/Cas9 IVTs or RNPs, particularly the latter, therefore drastically reduces off-target effects[39,40].

Time savings and low cost. Removing integrated foreign DNA by backcrossing in the conventional CRISPR/Cas9 DNA genome editing method takes a long time, especially in perennials. On the other hand, using CRISPR/Cas9 RNPs and IVTs, edited plants free of foreign DNA are generated in the T0 generation. This saves a lot of time and expense.

Valuable for specific plants. The DNA-free methods are particularly valuable for specific plants, such as transformation-recalcitrant and vegetatively propagated plants. For transformation-recalcitrant plants, there is no need to develop constructs with appropriate promoters for driving Cas9 and sgRNAs, and to identify efficient selective markers. For vegetatively propagated plants, it is very difficult or even impossible to segregate out the integrated foreign DNA fragments.

However, these methods still have some limitations:

Low efficiency. The mutation frequency achieved with CRISPR/Cas9 IVTs is slightly lower than that obtained with CRISPR/Cas9 DNA or RNPs. This may be due to the low intracellular concentrations of Cas9 and sgRNA obtained. Because no antibiotic selection is imposed during culture, many more regenerated plants must be tested than in the conventional DNA method. Thus, mutant screening is much more laborious.

Low stability of IVTs. mRNA is less stable than DNA or protein, as ubiquitous RNAases can accelerate its degradation if it is not properly protected; this imposes the need for particular skill in handling IVTs. Their instability also creates difficulties in evaluating their effectiveness in protoplasts, a procedure that is usually performed in an open lab environment.

Alternative methods

We have developed another transgene-free method using the same selection-free strategy. It is called transient expression of CRISPR/Cas9 DNA (TECCDNA)[39], and is much easier for novices who lack experience in handling *in vitro* synthesis of RNA or protein expression. In this alternative method, we deliver CRISPR/Cas9 plasmids into immature wheat embryos, but tissue culture takes place in the absence of antibiotics, unlike in the conventional method. This TECCDNA method has been proven for six target genes in bread wheat (cultivar Kenong199 and Bobwhite); mutants with targeted mutations, but no CRISPR/Cas9 constructs, were easily identified in T0 seedlings, and PCR analysis revealed that the percentage of transgene-free mutants could reach 86.8%. TECCDNA is easier to handle than CRISPR/Cas9 IVTs and RNPs, but has two main disadvantages: some off-target events with one mismatch still occurred, and it was possible that small CRISPR/Cas9 DNA fragments were integrated into unknown genomic regions and could not be easily detected.

Extending applications

We have also successfully used TECCDNA to knock out *TdGASR7* in two tetraploid durum wheat varieties (cultivar Shimai11 and Yumai4 of *T. turgidum* L. var. durum, AABB, $2n = 4x = 28$) (ref. 39). Therefore, theoretically the two DNA-free editing protocols described here could be adapted to knock out genes in other wheat species, such as tetraploid durum wheat. Moreover, Svitashev *et al.*[42] have reported success using biolistic delivery of CRISPR/Cas9 RNPs into embryo cells of maize and were able to regenerate plants in nonselective culture conditions. All of these results indicate that these DNA-free editing methods with CRISPR/Cas9 IVTs or RNPs should be applicable to different crops species and provide reliable genome editing for crop improvement.

Experimental design

Here we provide two step-by-step protocols for targeted mutagenesis in wheat using CRISPR/Cas9 IVTs or RNPs, respectively.

sgRNA design, cloning and validation. Due to the lack of a fine-scale genome database in wheat, no web tools are yet available. We usually select the sgRNA target sites manually based on the criterion that a 5′-NGG protospacer adjacent motif (PAM) is present directly downstream of the target sequence. We can use an sgRNA

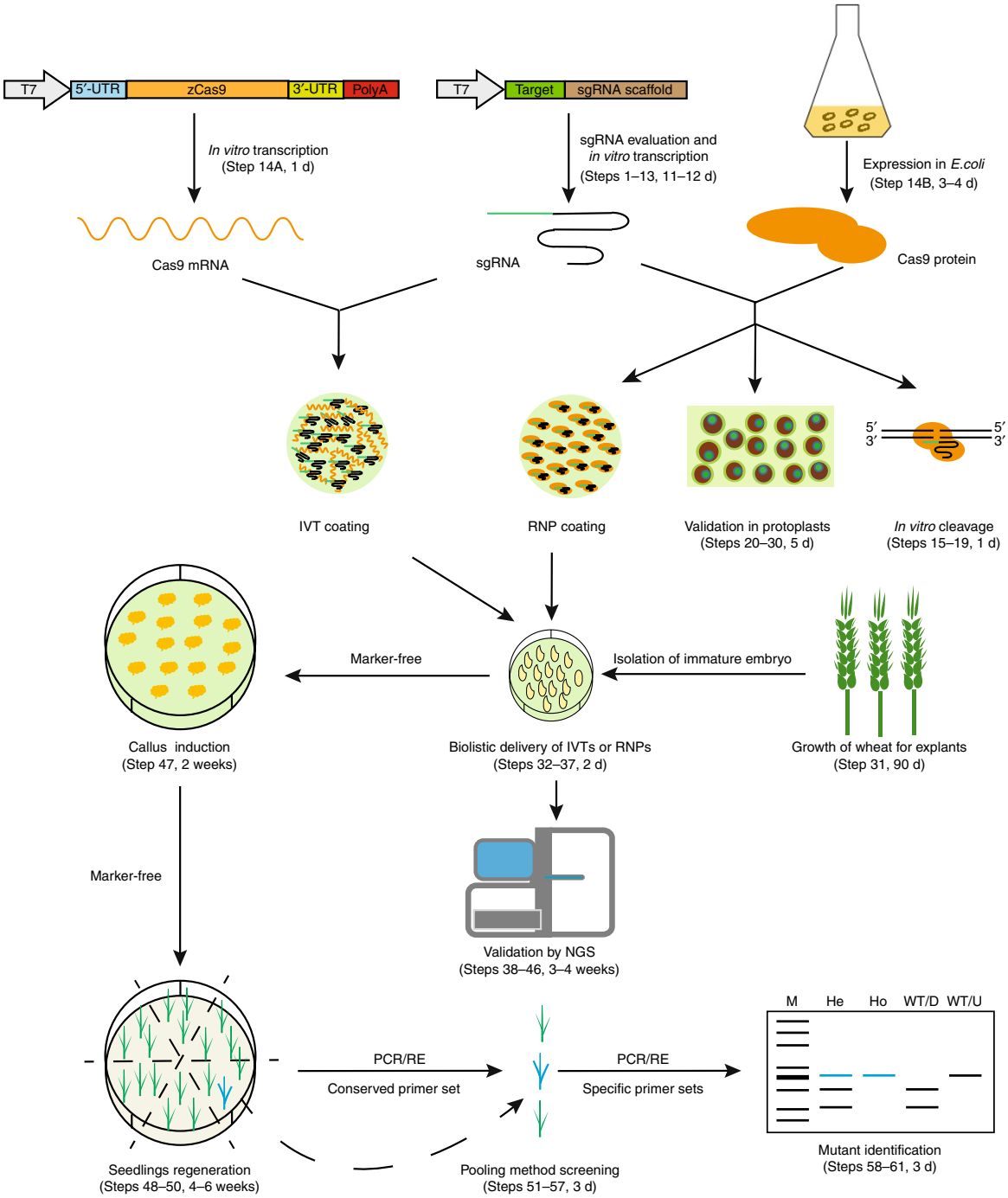

Figure 1 | Overview of CRISPR/Cas9 IVT- or RNP-mediated genome editing in wheat. Preparation of CRISPR/Cas9 IVTs and RNPs, coating, delivery, seedling regeneration and mutant screening are illustrated. Highly active candidate sgRNA is first evaluated in a transient protoplast assay and then transcribed using PCR products containing the T7 promoter upstream of the sgRNA sequence (Steps 1–13). Cas9 mRNA is transcribed using XbaI-linearized pLZT7-zCas9, which contains codon-optimized Cas9 flanked by two NLS sequences, the 5′- and 3′-untranslated regions of the maize Ubiquitin1 gene and a PolyA signal (Step 14A). Cas9 protein is induced, expressed and purified from bacterial cells (Step 14B). Preassembled RNP complexes are validated by *in vitro* cleavage (Steps 15–19) and transient protoplast assays (Steps 20–30). Growth of plants to generate immature embryos in the greenhouse takes 90 d and should be initiated in advance (Step 31). The CRISPR/Cas9 IVTs or RNPs are then delivered into the harvested immature embryos (Steps 32–37), and delivery is confirmed by NGS (Steps 38–46; **Fig. 2**). No antibiotic is used during the whole tissue culture procedure. Embryogenic calli can be induced after 2 weeks (Step 47), and testable plantlets can be regenerated after another 4–6 weeks (Steps 48–50). Seedlings are first screened by pooling and PCR/RE with conserved primers that recognize the three homologs simultaneously (Steps 51–57). The blue plant indicates a representative mutant. Seedlings in a pool that gives a positive signal are checked by PCR/RE and sequencing with homolog-specific primer sets (Steps 58–61). Mutations introduced by NHEJ can result in uncleaved bands, indicated in blue. He, heterozygous mutants; Ho, homozygous mutants; WT/D, wild-type amplicons digested with a restriction enzyme; WT/U, wild-type amplicon not digested.

to target all three copies in the A, B and D genome simultaneously, or to target one of them specifically, as required. Detailed information can be found in our previously published protocols[38]. Then we use the CasOT tool, a Perl script for genome-wide potential off-target searching (download from http://casot.cbi.pku.edu.cn/), to assess the specificity of the sgRNA[43]. CasOT is a local tool, and we need the wheat genome database from IWGSC (https://urgi.versailles.inra.fr/download/iwgsc/). We recommend using sgRNAs that have at least three mismatches in relation to any other genome sequences.

For ease of operation, an sgRNA is first cloned into pTaU6-sgRNA for confirming its activity. Unlike in our previous protocols, in this protocol the AarI restriction enzyme sites in pTaU6-sgRNA are replaced with BbsI sites, because BbsI is more efficient and much cheaper. The 2×35S promoter is also replaced with the maize Ubiquitin 1 promoter to enhance Cas9 expression, and the construct is designated as pJIT163-Ubi-Cas9. We co-deliver the sgRNA and Cas9 expression cassettes into protoplasts to validate their nuclease activity. We generally design three sgRNAs for each gene, and use the most efficient one. For unclear reasons, the nuclease activities of sgRNA vary greatly. Newcomers can use GW2-sgRNA, a high-efficiency sgRNA we used before, as a positive control for this protocol.

Production and delivery of CRISPR/Cas9 IVTs. To enable *in vitro* transcription of Cas9 mRNA, we constructed a plasmid containing the T7 promoter, the 5′- and 3′-untranslated regions of the maize Ubiquitin1 gene and a PolyA signal, in that order, and named it pLZT7. Next, we inserted the codon-optimized Cas9 sequence flanked by two nuclear localization signals (NLSs) into this vector (yielding pLZT7-zCas9). The plasmid was then digested by XbaI downstream of the PolyA signal, so that T7 polymerase falls off the template after transcription (**Fig. 1**). For sgRNA transcription, we amplify sgRNA expression cassettes with T7-Spacer-F (containing the T7 promoter and an initial transcribed base G) and sgRNA-R as template (**Fig. 1**). After purification, the Cas9 mRNA and sgRNA is analyzed by gel electrophoresis.

For biolistic delivery of CRISPR/Cas9 IVTs, we use ammonium acetate and 2-propanol to precipitate the IVTs onto gold particles in a process that is different from that used for coating with DNA. We use 1 μg of Cas9 mRNA and 1 μg of sgRNA for each shot. This dosage of CRISPR/Cas9 IVTs is enough to induce homozygous mutations in common wheat in the T0 generation.

Production, validation and delivery of CRISPR/Cas9 RNPs. Previous studies have shown that CRISPR/Cas9 delivered as ribonucleoprotein can efficiently cause targeted mutagenesis and at the same time reduce off-target effects[33,44,45]. For *in vitro* expression of Cas9 protein, we cloned the Cas9 coding sequence flanked by two NLSs into the pET28a$^+$ vector, which contains a C-terminal His-tag for purification, and designated the product pET28a-Cas9-His. Production of *in vitro*-transcribed sgRNA is described above.

Unlike CRISPR/Cas9 IVTs, the effectiveness of RNP complexes can be easily tested by *in vitro* cleavage of PCR products containing the target site. We also recommend testing the activity of RNP complexes *in vivo* using the protoplast transient assay (**Fig. 1**). Genomic DNA can be extracted 2 d post transfection, detected by PCR/RE or T7EI assays, and confirmed by Sanger sequencing. Furthermore, off-target effects can be detected in the protoplast assays.

To generate CRISPR/Cas9 RNP complexes for particle bombardment, we premix 2 μg of Cas9 protein and 2 μg of sgRNA in 1× Cas9 reaction buffer in a total volume of 10 μl at room temperature (25 °C) for 10 min for each shot. The molar ratio of sgRNA to Cas9 protein was kept at ~4–5:1. The RNP complexes are then mixed with 5 μl of gold nanoparticles, spread onto a macrocarrier and allowed to air-dry for coating. We are confident that our protocol will work reproducibly for common wheat in other labs, if it is followed properly. But for divergent wheat genotypes or other species, some adjustments may be needed.

Verification of CRISPR/Cas9 reagent delivery by deep sequencing (Steps 38–46). Biolistic delivery of biomaterials other than DNA into plant cells is still new to many labs. Therefore, we recommend that newcomers use deep sequencing to detect mutations to confirm the RNP delivery (**Fig. 1**). 2 d after transformation, genomic DNA is extracted from ~100 bombarded embryos (generally from two shots). In the first-round PCR, the target regions are amplified by homolog-specific primer sets that flank the sgRNA. The homolog-specific primer sets are designed to amplify 350- to 1,000-bp amplicons that contain the target site in or near the middle of the amplicon (**Table 1**). In the second-round PCR, for Hiseq-PE150 sequencing, the amplicons should be 110–130 bp. Barcodes are added onto both ends of the DNA fragments using primers with built-in barcode sequences. Thus, the forward and reverse barcoded PCR primers each contain a unique 6-nt barcode along with 20-nt site-specific sequences (**Fig. 2** and **Table 1**). The barcode sequences correspond to NEXTflex RNA-seq barcodes, which provide 48 available unique 6-nt barcodes. The barcodes can be used to distinguish the amplicons from the three different homologs or those from different treatments (such as DNA, IVTs or RNPs). Generally, the nonhomologous end joining (NHEJ) repair process results in variable lengths of indels at the target sites. To cover most of the indels, the designed 20-nt site-specific sequences should ensure that the sgRNA is located in the middle of the amplicons. Afterward, the amplicons can be mixed as a pool for next-generation sequencing. In the pool, different target sites can be mixed and sequenced simultaneously. For each target site, as many as 24 PCR products can be distinguished by the barcodes. Different target sites can be distinguished by barcodes along with target-specific sequence. For each PCR product, ~100,000 reads

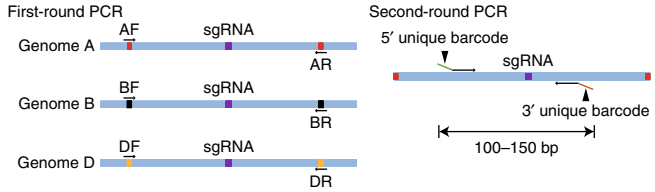

Figure 2 | Schematic of PCR strategies for next-generation sequencing of bombarded immature embryos to confirm CRISPR/Cas9 delivery (Steps 38–46). In the first round of PCR, homolog-specific primers are designed to flank the target site (300- to 800-bp region), and barcoded amplicons (100–150 bp) are obtained in the second round. Forward and reverse primers are designed with a unique 6-nt barcode along with a 20-nt site-specific sequence. AF, forward homoeolog-specific primer for A genome; AR, reverse homoeolog-specific primer for A genome; BF, forward homoeolog-specific primer for B genome; BR, reverse homoeolog-specific primer for B genome; DF, forward homoeolog-specific primer for D genome; DR, reverse homoeolog-specific primer for D genome.

TABLE 1 | Primer sequences used in this protocol.

Step	Name	Sequence (5′–3′)	Purpose
2	T7-spacer-F	TAATACGACTCACTATAGGNNNNNNNNNNNNNNNNNNN	Forward primer to amplify the sgRNA template
2	gRNA-R	GCACCGACTCGGTGCCACTT	Reverse primer to amplify the sgRNA template
15, 30	CER9-F	TACTGGCATGGATGACGCTAC	Forward primer to amplify the *TaCER9* target site
15, 30	CER9-R	CAAGTGCCCAATCCTGATACA	Reverse primer to amplify the *TaCER9* target site
30	LOX2-F	CGTCTACCGCTACGACGTCTACAACG	Forward primer to amplify the *TaLOX2* target site
30	LOX2-R	GGTCGCCGTACTTGCTCGGATCAAGT	Reverse primer to amplify the *TaLOX2* target site
40	GW2-A1-F	CTGCCATTACTTTGTATTTTGGTAATA	Forward primer to amplify the *TaGW2-A1* target site for first-round PCR
40	GW2-B1-F	GTTCAGATGGCAATCTAAAAGTT	Forward primer to amplify the *TaGW2-B1* target site for first-round PCR
40	GW2-D1-F	GCATGTACTTTGATTGTTTGCGTGA	Forward primer to amplify the *TaGW2-D1* target site for first-round PCR
40	GW2-A1/B1/D1-R	TCCTTCCTCTCTTACCACTTCCC	Reverse primer to amplify the *TaGW2-A1*, *TaGW2-B1* and *TaGW2-D1* target sites for first-round PCR
42	BA1-GW2-AF	CGATGTTGCCTTTTGAGCAACCAACG	Barcoded forward primer to amplify the *TaGW2-A1* target site for second-round PCR
42	BA2-GW2-AR	TGACCATCCATGCTTGATGGCTGGTG	Barcoded reverse primer to amplify the *TaGW2-A1* target site for second-round PCR
42	BA3-GW2-BF	ACAGTGTGCCTTTTGAGCAACCAACG	Barcoded forward primer to amplify the *TaGW2-B1* target site for second-round PCR
42	BA4-GW2-BR	GCCAATTCCATGCTTGATGGCTGGTG	Barcoded reverse primer to amplify the *TaGW2-B1* target site for second-round PCR
42	BA5-GW2-DF	CAGATCTGCCTTTTGAGCAACCAACG	Barcoded forward primer to amplify the *TaGW2-D1* target site for second-round PCR
42	BA6-GW2-DR	CTTGTATCCATGCTTGCTGGCTGGTG	Barcoded reverse primer to amplify the *TaGW2-D1* target site for second-round PCR
53, 59	GASR7-F	GGAGGTGATGGGAGGTGGGGG	Conserved forward primer to amplify the *TaGASR7* target site
53, 59	GASR7-R	CTGGGAGGGCAATTCACATGCCA	Conserved reverse primer to amplify the *TaGASR7* target site
60, 61	GASR7-A1/B1/D1-F	CCTTCATCCTTCAGCCATGCAT	Homoeolog-specific forward primer to amplify the *TaGASR7-A1*, *TaGASR7-B1* and *TaGASR7-D1* target sites
60, 61	GASR7-A1-R	CCACTAAATGCCTATCACATACG	Homoeolog-specific reverse primer to amplify the *TaGASR7-A1* target site
60, 61	GASR7-B1-R	AGGGCAATTCACATGCCACTGAT	Homoeolog-specific reverse primer to amplify the *TaGASR7-B1* target site
60, 61	GASR7-D1-R	CCTCCATTTTTCCACATCTTAGTCC	Homoeolog-specific reverse primer to amplify the *TaGASR7-D1* target site

are enough to detect the mutations. Mutagenesis frequency is calculated as the percentage of reads containing indels in the target site over the total reads sequenced.

Mutation recovery and genotyping (Steps 51–61). For the DNA-free genome editing methods in wheat, we use no selectable markers throughout the entire tissue culture procedure. The selection-free procedure takes only 6–8 weeks after particle bombardment to obtain regenerated plants, which is 2–4 weeks less than those of previous editing methods (**Fig. 1**).

Sample pooling for screening regenerated plantlets. As hundreds of plantlets are usually obtained under selection-free conditions, we use sample pooling to screen them in order to save labor, time and cost. Three to four plantlets, regenerated from the same bombarded embryo, are labeled numerically, with a piece of leaf (~10 × 3 mm) taken from each of them aseptically. The leaf pieces are combined as one pool for genomic DNA extraction and subsequent screening (**Fig. 1**). A PCR/RE assay is conducted with a conserved primer set that recognizes all three homologs[38] (**Table 1**). The conserved primers are designed to amplify 350- to 1,000-bp amplicons that contain the target site in or near the middle of the amplicon. After agarose gel electrophoresis, the pools with undigested bands are the ones containing mutants. Next, the seedlings in the mutant-containing pools are transferred to new rooting medium to grow for 1 week. They are then individually sampled and screened by PCR/RE assay as above. The positive seedlings are further screened by PCR with homolog-specific primer sets, thus revealing the mutagenesis in each homolog (**Fig. 1**).

PCR/RE assays. To save labor and costs, we strongly recommend using PCR/RE assays for screening for mutants. An essential requirement is the presence of a restriction enzyme site in the target sequence[38]. The indels induced by sgRNA:Cas9 complexes may destroy the restriction enzyme site and lead to uncleaved bands after digestion, and previous studies have shown that the most frequent indels are 1-bp insertions or deletions at the target site[46]. Therefore, in order to avoid missing these types of mutations, sgRNAs that contain restriction sites that just cover the Cas9 cutting site (3 bp upstream of the PAM sequence) are preferred. For PCR/RE assays in hexaploid wheat, we first design a conserved primer set that recognizes all three homologs, and then three specific primer sets, one for each homolog. Subsequently, we perform Sanger sequencing to identify the types of mutation.

T7EI assays. T7EI can serve as an alternative nuclease for targets in which no suitable restriction site is available. T7EI is a mismatch-specific nuclease that cleaves heteroduplex (imperfectly matched) DNA. For T7EI assays in hexaploid wheat, homolog-specific primer sets are needed, because the different alleles may contain single-nucleotide polymorphisms in the amplified region, which can affect the accuracy of T7EI cutting. Furthermore, T7EI can be used only to detect heterozygous and bi-allelic mutants, as it cannot distinguish homozygous mutants from the wild type. Mutants should be further confirmed by Sanger sequencing.

Amplicon sequencing. Furthermore, direct amplicon sequencing can also be adopted for mutant identification. However, direct amplicon sequencing in hexaploid species usually requires homolog-specific primer sets, which are more costly and laborious than first using PCR/RE to screen with a conserved primer set.

Controls. For newcomers to genome editing in wheat, we suggest using gw2-sgRNA as a positive control, as we have shown it to be highly effective in targeted mutagenesis (**Supplementary Table 1**). In protoplasts, the on-target mutagenesis frequencies of *GW2-B1* and *GW2-D1* induced by gw2-RNPs were 33.4 and 21.8%, respectively. The gw2-RNPs yielded a mutation frequency of ~0.2% in immature embryos 2 d post bombardment. In the T0 generation, gw2-IVTs and gw2-RNPs had MPEs (mutant production efficiencies) of ~1.1 and 4.4%, respectively.

In addition, using the TECCDNA method in parallel can serve as a control, because plasmids are much easier to handle than CRISPR/Cas9 IVTs or RNPs. TECCDNA editing with plasmids of gw2-sgRNA (pGE-TaGW2) (ref. 39), which we have shown to be highly active, could be used as a control to determine transformation efficiencies and mutation frequencies.

MATERIALS

REAGENTS

Wheat cultivars

- Kenong 199 (available from the authors upon request)
- YZ814 (available from the authors upon request)

Plasmids

▲ **CRITICAL** All plasmids can be obtained from the authors upon request.

- pJIT163-Ubi-Cas9, for Cas9 expression in wheat. See **Supplementary Note 1** for the full sequence.
- pLZT7-zCas9, for *in vitro* transcription of Cas9 mRNA. See **Supplementary Note 2** for the full sequence.
- pET28a-Cas9-His, for *in vitro* expression of Cas9 protein. See **Supplementary Note 3** for the full sequence.

Production of CRISPR/Cas9 IVTs

- PCR primers (from BGI) are listed in **Table 1** and in **Supplementary Table 1**.
- TransStart FastPfu DNA polymerase (TransGen Biotech, cat. no. AP221-03)
- High Pure dNTPs (2.5 mM each; TransGen Biotech, cat. no. AD101-02)
- TAE buffer (10×; Cellgro, cat. no. 46-010-CM)
- Agarose (Invitrogen, cat. no. 16500500)
- EasyPure PCR Purification Kit (TransGen Biotech, cat. no. EP101-01)
- 10× Fastdigest Green Buffer (Fermentas/Thermo Scientific, cat. no. B72)
- GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Fisher Scientific, cat. no. k0841)
- AxyPrep DNA gel extraction kit (Axygen, cat. no. AP-GX-250)
- HiScribe T7 High Yield RNA Synthesis Kit (NEB, cat. no. E2040S)
- FastDigest XbaI (Fermentas/Thermo Scientific, cat. no. FD0685)
- mMESSAGE mMACHINE T7 Kit (Ambion, cat. no. AM1344)
- MEGAclear Transcription Clean-Up Kit (Ambion, cat. no. AM1908)
- Ethanol (Sigma-Aldrich, cat. no. E7023)
- RNase-free H_2O (Amresco, cat. no. E476)
- RNase-free 1.5-ml microcentrifuge tubes (Axygen, cat. no. MCT-150-C)

Production of Cas9 protein

- Kanamycin (50 mg/ml, sterile filtered; Genview, cat. no. AK177)
- Isopropyl-β-d-1-thiogalactopyranoside (IPTG; Inalco, cat. no. 1758-1400)
- Imidazole (Sigma-Aldrich, cat. no. I5513)
- Trizma base (Sigma-Aldrich, cat. no. T1503)
- 30% (wt/vol) Acrylamide solution (Applygen, cat. no. B1000)
- Ammonium persulfate (Sigma-Aldrich, cat. no. A3678)
- Ni-sepharose 6 Fast Flow (GE Healthcare, cat. no. 17-5318-01)
- Quick Start Bradford 1× dye reagent (Bio-Rad, cat. no. 5000205)
- Glycine (Sigma-Aldrich, cat. no. 50046)
- SDS (Amresco, cat. no. L3771)
- KCl (Sigma-Aldrich, cat. no. P5405)
- NaCl (Sigma-Aldrich, cat. no. S3014)
- Transetta (DE3) chemically competent cells (TransGen Biotech, cat. no. CD801-02)

- BL21 (DE3) chemically competent cells (TransGen Biotech, cat. no. CD601-02)
- HEPES (Sigma-Aldrich, cat. no. 54457)
- DTT (Sigma-Aldrich, cat. no. 43816)
- Glycerol (Amresco, cat. no. 0854)
- $MgCl_2$ (Sigma-Aldrich, cat. no. 208337)
- Na_2HPO_4 $12H_2O$ (Sigma-Aldrich, cat. no. 04273)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P9791)

Protoplast isolation and transformation

- 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma-Aldrich, cat. no. M8250)
- Mannitol (Amresco, cat. no. 0122)
- $CaCl_2{\cdot}2H_2O$ (Sigma-Aldrich, cat. no. C7902)
- KCl (Sigma-Aldrich, cat. no. P3911)
- BSA (Sigma-Aldrich, cat. no. A-6793)
- $MgCl_2$ (Sigma-Aldrich, cat. no. M9272)
- Cellulase R-10 (Yakult Pharmaceutical Ind.)
- Macerozyme R-10 (Yakult Pharmaceutical Ind.)
- PEG 4000 (Sigma-Aldrich, cat. no. 95904)

Particle bombardment and tissue culture

- Gold nanoparticles (Bio-Rad, cat. no. 165-2262)
- Ammonium acetate (Sigma-Aldrich, cat. no. A1542)
- 2-propanol (Sigma-Aldrich, cat. no. I9516)
- Murashige and Skoog (MS) medium basal salt mixture (Phytotech, cat. no. M524)
- Murashige and Skoog (MS) salt, including vitamins (Phytotech, cat. no. M519)
- Phytagel (Sigma-Aldrich, cat. no. P8169-5Kg)
- Sucrose (Sigma-Aldrich, cat. no. V900116)
- 2,4-Dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich, cat. no. D7299)
- Kinetin (Sigma-Aldrich, cat. no. K0753)
- N-Z-Amine A (Sigma-Aldrich, cat. no. C7290)
- $CuSO_4{\cdot}5H_2O$ (Sigma-Aldrich, cat. no. C3036)
- KOH (Sigma-Aldrich, cat. no. 484016)
- 1-Naphthaleneacetic acid (NAA; Sigma-Aldrich, cat. no. N0640)

PCR/RE and T7EI assays

- DNA Quick Plant System (Tiangen Biotech, cat. no. DP321-03)
- Cetyltrimethyl ammonium bromide (CTAB; Sigma-Aldrich, cat. no. H6269)
- 2× Taq MasterMix (CWBIO, cat. no. CW0960A)
- FastDigest XbaI (Fermentas/Thermo Scientific, cat. no. FD0685)

EQUIPMENT

- PCR thermocycler (Veriti 96-well thermal cycler; Applied Biosystems, cat. no. 9902)
- Heating water bath (Memmert, cat. no. WNB22L4L5)
- UV-visible light spectrophotometer (Thermo, model no. NanoDrop ND-2000)
- Centrifuge series (Eppendorf, model nos. 5810R, 5424 and 5417R)
- High-speed centrifuge (Beckman Coulter, model no. Avanti JXN-30
- Incubator shaker (Eppendorf, model no. Innova42)
- Polypropylene gravity-flow purification column (Bio-Rad, cat. no. 732-1010)
- Amicon Ultra-15 centrifugal filter units (Millipore, cat. no. UFC903024)
- Standard equipment and reagents for SDS-PAGE gel electrophoresis (Bio-Rad)
- Standard equipment and reagents for agarose gel electrophoresis (Bio-Rad)
- Fluorescence microscope (Olympus, model no. SZX16)
- PDS1000/He particle bombardment system (Bio-Rad)
- Digital gel imaging system (BioDoc-It; UVP, cat. no. 97-0256-02)
- Gel quantification software (ImageJ, NIH, http://rsbweb.nih.gov/ij/)
- High-throughput tissue homogenizer (SPEX SamplePrep, 2010 Geno/Grinder)
- Sonic dismembrator (Fisher Scientific, cat. no. F 550)
- 50-ml Round-bottom centrifuge tubes (Haimeng, cat. no. LXG-50Y)
- Single-edge razor blade (Feiying, cat. no. 9875814)
- 40-μm Nylon mesh (BD Falcon, cat. no. F613461)
- 0.45-μm Syringe sterilization filter (Sartorius Stedim Biotech, cat. no. 21423103)
- Petri dish (Greiner Bio-One, cat. no. 633180)

REAGENT SETUP

LB medium (1 liter). Dissolve 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in dH_2O. Adjust the volume to 1 liter with RNase-free water and the pH to 7.5, and then autoclave. Store at 4 °C for up to 1 month.

LB agar plates (1 liter). Add 14 g of agar to 1 liter of LB medium. Sterilize by autoclaving and add appropriate antibiotic after cooling to ~60 °C. Pour ~30 ml of LB agar per sterile Petri dish. Store at 4 °C for up to 1 month.

2× YT medium (1 liter). Dissolve 10 g of tryptone, 10 g of yeast extract and 5 g of NaCl in dH_2O. Adjust the volume to 1 liter with RNase-free water and the pH to 7.5, then autoclave. Store at 4 °C for up to 1 month.

3 M KCl (stock solution, 15 ml). Add 3.35 g of KCl to RNase-free water and adjust the volume to 15 ml with RNase-free water. Store at room temperature for up to 1 year.

1 M HEPES (stock solution, 10 ml). Add 2.38 g of HEPES to RNase-free water and adjust the volume to 10 ml with RNase-free water and the pH to 7.5 with NaOH. Store at 4 °C for up to 6 months.

1 M DTT (stock solution, 10 ml). Add 1.54 g of DTT to RNase-free water and adjust the volume to 10 ml with RNase-free water. Prepare 1-ml aliquots and store at −20 °C for up to 6 months.

1 M $MgCl_2$ (stock solution, 10 ml). Add 2.03 g of $MgCl_2{\cdot}6H_2O$ to RNase-free water and adjust the volume to 10 ml with RNase-free water. Store at room temperature for up to 1 year.

1 M $CaCl_2$ (stock solution, 100 ml). Add 14.7 g of $CaCl_2{\cdot}2H_2O$ to RNase-free water and adjust the volume to 10 ml with RNase-free water. Store at room temperature for up to 1 year.

200 mM MES (stock solution, 10 ml). Add 0.39 g of HEPES to RNase-free water, and adjust the volume to 10 ml with RNase-free water and the pH to 5.7 with NaOH. Store at 4 °C for up to 6 months.

5 M Ammonium acetate (10 ml). Add 3.85 g of ammonium acetate to RNase-free water and adjust the volume to 10 ml with RNase-free water. Store at −20 °C for up to 1 year.

0.5 M IPTG (10 ml). Add 1.19 g of IPTG to ddH_2O. Adjust the volume to 10 ml with RNase-free water. Sterilize with a 0.22-μm filter and store at −20 °C for up to 1 year.

10× PBS buffer (1 liter). Add 80 g of NaCl, 2 g of KCl, 35.8 g of Na_2HPO_4 $12H_2O$ and 2.4 g of KH_2PO_4 to ddH_2O. Adjust the volume to 1 liter with RNase-free water. Store at room temperature for up to 6 months.

1 M Tris (1 liter, pH 8.0). Add 121.14 g of Trizma base to ddH_2O. Adjust the volume to 1 liter with RNase-free water and the pH to 8.0 with HCl. Store at room temperature for up to 6 months.

1 M Tris (100 ml, pH 7.5). Add 12.11 g of Trizma base to ddH_2O. Adjust the volume to 100 ml with RNase-free water and the pH to 7.5 with HCl. Store at room temperature for up to 6 months.

5 M NaCl (100 ml). Add 29.22 g of NaCl to ddH_2O. Adjust volume to 100 ml with RNase-free water. Store at room temperature for up to 6 months.

Buffer I (1 liter). Add 25 ml of 1 M Tris (pH 8.0) and 29.22 g of NaCl to ddH_2O. Adjust the volume to 1 liter with RNase-free water. Store at 4 °C for up to 6 months.

Buffer E (500 ml). Add 12.5 ml of 1 M Tris (pH 8.0), 50 ml of 5 M NaCl and 34.04 g of imidazole to ddH_2O. Adjust the volume to 500 ml with RNase-free water. Store at 4 °C for up to 6 months.

Lysis buffer (100 ml). Add 2.5 ml of buffer E to 97.5 ml of buffer I. Store at 4 °C for up to 6 months.

Wash buffer (100 ml). Add 5 ml of buffer E to 95 ml of buffer I. Store at 4 °C for up to 6 months.

Elution buffer (100 ml). Add 25 ml of buffer E to 75 ml of buffer I. Store at 4 °C for up to 6 months.

Cas9 storage buffer (100 ml). Add 2 ml of 1 M HEPES, 5 ml of 3 M KCl, 100 μl of 1 M DTT and 3 ml of glycerol to autoclaved ddH_2O. Adjust the volume to 100 ml with RNase-free water. Store at 4 °C for up to 6 months.

Buffer D (100 ml). Add 25 ml of 1 M Tris (pH 7.5), 6.67 ml of 3 M KCl, 1 ml of 1 M $MgCl_2$ and 3 ml of glycerol to autoclaved ddH_2O. Adjust the volume to 100 ml with RNase-free water. Store at 4 °C for up to 6 months.

10× Cas9 reaction buffer (10 ml). Add 2 ml of 1 M HEPES, 1 ml of 1 M $MgCl_2$, 50 μl of 1 M DTT and 5 ml of 3 M KCl to RNase-free water. Adjust the volume to 10 ml with RNase-free water. Prepare 1-ml aliquots and store at −20 °C for up to 1 year.

10% (wt/vol) Ammonium persulfate (10 ml). Add 1 g of ammonium persulfate to ddH_2O. Adjust the volume to 10 ml with RNase-free water. Store at 4 °C for up to 2 months.

10× Running buffer (1 liter). Add 30.3 g of Trizma base, 144 g of glycine and 10 g of SDS to ddH_2O. Adjust the volume to 1 liter with RNase-free water. Store at room temperature for 6 months.

0.8 M Mannitol (20 ml). Add 2.91 g of mannitol to RNase-free water. Adjust the volume to 200 ml with RNase-free water. Sterilize with a 0.45-μm filter and store at room temperature for up to 2 months.

Enzyme solution (50 ml). Add 0.75 g of Cellulase R-10, 0.375 g of macerozyme R-10, 5.465 g of mannitol and 0.098 g of MES to RNase-free water. Adjust the volume to 50 ml with RNase-free water and the pH to 5.7 with 1 M KOH. 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 and add 500 µl of 1 M $CaCl_2$ and 0.05 g of BSA. Finally, sterilize the solution with a 0.45-µm filter. The enzyme solution should be freshly prepared.

Plant growth conditions. Grow Kenong199 seeds at 25 °C with 16 h light/8 h dark for 7–14 d.

W5 solution (500 ml). Add 4.5 g of NaCl, 9.189 g of $CaCl_2{\cdot}2H_2O$, 0.1864 g of KCl and 0.1952 g of MES to ddH_2O. Adjust the volume to 500 ml with RNase-free water and the pH to 5.7 with NaOH, and then autoclave. Store at room temperature for up to 2 months.

MMG solution (10 ml). Add 5 ml of 0.8 M mannitol, 150 µl of 1 M $MgCl_2$ and 200 µl of 200 mM MES to RNase-free water. Adjust the volume to 10 ml with RNase-free water. Store at room temperature for up to 3 d.

PEG solution (4 ml). Add 4 g of PEG, 1 ml of 0.8 M mannitol and 400 µl of 1 M $CaCl_2$ to RNase-free water. Adjust the volume to 4 ml with RNase-free water. This solution should be freshly prepared.

Gold nanoparticles (1.5 ml). Add 0.06 g of gold (60-µm diameter) to RNase-free water and adjust the volume to 1.5 ml with RNase-free water.

Osmotic medium (1 liter). Add 4.4 g of Murashige and Skoog (MS) medium basal salt mixture, 5 ml of 2,4-D (1 mg/ml) and 72.867 g of mannitol to 800 ml ddH_2O. Adjust the volume to 1 liter with RNase-free water and the pH to 5.8 with 1 M KOH, and add 3.2 g of phytagel. Autoclave at 121 °C for 20 min. After cooling to 50 °C, pour ~20 ml per sterile Petri dish. Store the dishes at 4 °C for up to 6 weeks in dark containers.

Recovery medium (1 liter). Add 4.4 g of Murashige and Skoog (MS) salt including vitamins, 30 g of sucrose, 2 ml of 2,4-D (1 mg/ml), 0.5 g of N-Z-Amine A and 600 µl of $CuSO_4$ (1 mg/ml) to 800 ml of ddH_2O. Adjust the volume to 1 liter with RNase-free water and the pH to 5.8 with 1 M KOH, and add 3.2 g of phytagel. Autoclave at 121 °C for 20 min. Pour ~30 ml into sterile Petri dishes after cooling to 50 °C. Store dishes at 4 °C for up to 6 weeks in dark containers.

Regeneration medium (1 liter). Add 4.4 g of Murashige and Skoog (MS) salts including vitamins, 30 g of sucrose and 200 µl of kinetin (1 mg/ml) to 800 ml of ddH_2O. Adjust the volume to 1 liter with RNase-free water and the pH to 5.8 with 1 M KOH, and add 3.2 g of phytagel. Autoclave at 121 °C for 20 min. Pour ~30 ml of medium into sterile Petri dishes after cooling to 50 °C. Store the dishes at 4 °C for up to 6 weeks in dark containers.

Rooting medium (1 liter). Add 2.2 g of Murashige and Skoog (MS) salts including vitamins and 30 g of sucrose to 800 ml of ddH_2O. Adjust the volume to 1 liter with RNase-free water and the pH to 5.8 with 1 M KOH, and add 3.2 g of phytagel. Autoclave at 121 °C for 20 min. After cooling to ~50 °C, add 100 µl of NAA (0.5 mg/ml). Pour ~30 ml of medium into sterile Petri dishes. Store the 4 °C for up to 6 weeks in dark containers.

PROCEDURE

sgRNA cloning and evaluation by transient protoplast assays ● TIMING ~10 d

1| Clone and validate sgRNAs using detailed protocols found in our previous publication[38] (see also the Experimental design section for further details). One improvement is that we recommend pJIT163-Ubi-Cas9 for Cas9 expression to enhance expression levels in protoplasts.

? TROUBLESHOOTING

Production of *in vitro*-transcribed sgRNA ● TIMING 1–2 d

2| PCR-amplify the previously-cloned sgRNA expression cassette using T7-spacer-F and gRNA-R in two 50-µl reactions as follows:

Component	Amount (µl)	Final concentration
FastPfu buffer, 5×	10	1×
dNTP, 10 mM (2.5 mM each)	5	1 mM
T7-spacer-F, 10 µM	2	0.4 µM
gRNA-R, 10 µM	2	0.4 µM
FastPfu polymerase	1	
Plasmid template (from Step 1)	1	0.1–0.2 ng/µl
ddH_2O	Up to 50	

3| Perform the PCR reactions using the following conditions:

Cycle number	Denaturation	Annealing	Extension
1	95 °C, 2 min		
2–36	95 °C, 20 s	60 °C, 20 s	72 °C, 20 s
37			72 °C, 5 min

4| Run 5 µl of the PCR products on a 1.2% (wt/vol) agarose gel in TAE buffer at 140 V for 15 min. A single 114-bp band is expected.

5| Purify the PCR products using an EasyPure PCR Purification Kit according to the manufacturer's instructions. Measure the concentration with a NanoDrop spectrophotometer; a concentration >60 ng/µl is needed for *in vitro* transcription.
▲ **CRITICAL STEP** RNase-free water must be used for elution.

6| In vitro *transcription*. Set up the reaction using a HiScribe T7 High-Yield RNA Synthesis Kit (NEB) Kit, and assemble the reaction at room temperature in the following order: Mix gently and thoroughly by pipetting and incubate at 37 °C in the PCR machine for 2–3 h.

Component	Amount (µl)
RNase-free water	Up to 20
10× Reaction buffer	2
ATP (100 mM)	2
GTP (100 mM)	2
UTP (100 mM)	2
CTP (100 mM)	2
Template DNA (from Step 5)	(0.5–1 µg)
T7 RNA polymerase mix	2

7| Add 1 µl of DNase I and incubate at 37 °C for 1 h to digest the template DNA.
▲ **CRITICAL STEP** Incubation for longer can completely degrade the template DNA.

8| Transfer the mixture to a new RNase-free 1.5-ml microcentrifuge tube. Add 130 µl of RNase-free water, mix thoroughly and then add 300 µl of ethanol. Incubate the mixture at −20 °C for 3 h or overnight.
■ **PAUSE POINT** Incubating overnight may give a higher yield of sgRNA.

9| Centrifuge the samples at 12,000*g* for 10 min at 4 °C and discard the supernatant.

10| Wash the precipitate with 600 µl of 70% (vol/vol) ethanol and repeat Step 9.
▲ **CRITICAL STEP** Prepare the 70% ethanol with RNase-free water.

11| Centrifuge again at 12,000*g* for 2 min at 4 °C and remove the residual supernatant with a pipette.

12| Air-dry the pellet for 2 min at room temperature.

13| Add 50 µl of RNase-free water to dissolve the precipitate; then determine the RNA concentration with the NanoDrop spectrophotometer. Make 10-µl aliquots of purified sgRNA in 1.5-ml microcentrifuge tubes for single use and store at −80 °C for further use in Step 37A(i) or 37B(i).
? TROUBLESHOOTING
■ **PAUSE POINT** Purified sgRNA can be stored at −80 °C for 2 months.

Production of DNA-free Cas9 reagents
14| Use option A to produce *in vitro*-transcribed Cas9 mRNA to be used in Step 37A(i) for the biolistic delivery of CRISPR/Cas9 IVTs, or follow option B to produce Cas9 protein to be used in Step 37B(i) for the biolistic delivery of CRIPSR/Cas9 RNPs.

(A) *In vitro* transcription of Cas9 mRNA ● TIMING 1 d

▲ **CRITICAL STEP** Cas9 mRNA is commercially available. However, Cas9 mRNA prepared with plasmids as described below is codon-optimized and contains the 5- and 3-UTR regions of the maize Ubiquitin1 gene. This may facilitate the translation of Cas9 protein in plant cells, and therefore increase the mutagenesis frequency.

(i) To linearize pLZT7-zCas9 with XbaI, set up the reaction as follows: Incubate the reaction at 37 °C for 2 h.

Component	Amount (μl)
pLZT7-zCas9	(6 μg)
10× Fastdigest Green Buffer	15
XBaI	6
ddH_2O	Up to 150

(ii) Run 5 μl of digested plasmids on a 1.2% (wt/vol) agarose gel in TAE buffer at 140 V for 15 min. A single 8.3-kb band is expected.

(iii) Purify the remaining linearized plasmid using an EasyPure PCR purification kit. Measure the concentration with the NanoDrop spectrophotometer; a concentration >85 ng/μl is needed for *in vitro* transcription.

(iv) In vitro *transcription*. Set up the reaction using the mMESSAGE mMACHINE T7 Kit and assemble the reaction in order as follows: Gently mix by pipetting and incubate the reactions at 37 °C for 2 h.

Component	Amount (μl)
RNase-free water	Up to 20
2× NTP	10
10× Reaction buffer	2
Linearized pLZT7-zCas9	(0.5–1 μg)
Enzyme mix	2

▲ **CRITICAL STEP** Keep the 10× reaction buffer at room temperature after thawing and set up the reactions at room temperature.

(v) Add 1 μl of Turbo DNase from the kit and incubate at 37 °C for 15 min to remove the template DNA.

(vi) Purify the Cas9 mRNA using a MEGAclear Transcription Clean-Up Kit according to the manufacturer's instructions and elute with 50 μl of RNase-free water.

(vii) Determine the RNA concentration with the NanoDrop spectrophotometer. Usually the concentration is ~500 ng/μl. Store at −80 °C for later use.

▲ **CRITICAL STEP** Repeated freeze–thaw cycles may result in degradation of Cas9 mRNA. Make 10-μl aliquots of Cas9 mRNA in 1.5-ml microcentrifuge tubes for single use.

▲ **CRITICAL STEP** Due to the low yield of Cas9 mRNA, the CRISPR/Cas9 IVTs are not validated in protoplasts.

■ **PAUSE POINT** Purified Cas9 mRNA can be stored at −80 °C for 2 months.

(B) Production of Cas9 protein ● TIMING 3–4 d

▲ **CRITICAL STEP** Cas9 protein is commercially available. However, we have found that the commercial Cas9 protein contains more glycerol, which is difficult to air-dry for particle bombardment (Step 37B), and that the protein is sticky on the macro-carrier and cannot be delivered into the immature embryo cells efficiently.

(i) Add 50 ng of pET28a-Cas9-His plasmid to 50 μl of thawed *Transetta* (DE3) chemically competent cells on ice, mix gently and keep on ice for 30 min. Heat-shock at 42 °C for 45 s in a water bath and transfer to ice for 2 min. Add 500 μl of LB medium to the tube and incubate at 37 °C with shaking at 200 r.p.m. for 1 h.

▲ **CRITICAL STEP** Optionally, BL21 (DE3) can also be used for expressing Cas9.

(ii) Plate 100 μl of the transfected cells onto LB agar plates containing 50 μg/ml kanamycin and incubate at 37 °C overnight.

(iii) Pick a single colony into 10 ml of 2× YT medium containing 50 μg/ml kanamycin and incubate at 37 °C with shaking at 200 r.p.m. for 8 h.

(iv) Transfer the culture into 1 liter of 2× YT medium containing 50 µg/ml kanamycin and shake at 200 r.p.m. and 37 °C for 2–3 h (until the OD_{600} value reaches 0.6–0.8).
(v) Induce Cas9 expression with 0.5 mM IPTG and incubate at 18 °C with shaking at 200 r.p.m. for ~16 h.
(vi) Harvest the culture by centrifugation at 3,200*g* for 10 min at 4 °C.
(vii) Discard the supernatant and resuspend the cell pellet in precooled 1× PBS buffer. Centrifuge at 3,200*g* for 10 min at 4 °C.
(viii) Discard the supernatant and resuspend in 30 ml of lysis buffer and transfer to a fresh 50-ml centrifuge tube
(ix) Lyse the cells by sonication with 30% power output using a 10-min process time with cycles of 3 s on/6 s off.
(x) Centrifuge the lysate at 14,000*g* for 50 min at 4 °C. Transfer the supernatant to a fresh 50-ml collection tube.
▲ **CRITICAL STEP** To avoid crushing during high-speed centrifugation, use round-bottom tubes for centrifugation at 14,000*g*.
(xi) Add 1 ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Ni-sepharose 6 Fast Flow) to a gravity-flow purification column. Equilibrate the beads three times with 5 ml of lysis buffer.
(xii) Add the supernatant from Step 14B(x) to the column to bind the protein onto the column. Collect the flow-through and repeat this step three times for maximum binding of the protein.
(xiii) Wash the column with wash buffer to remove nonspecific binding proteins. Add 20 µl of flow-through to 200 µl of Quick Start Bradford 1× dye reagent to monitor this process until the color does not change. Usually ~50 column volumes of wash buffer are enough.
(xiv) Elute the Cas9 protein with elution buffer. Use Quick Start Bradford 1× dye reagent to monitor the flow-through until the color does not change, as described in Step 14B(xiii). Usually ~30 column volumes of elution buffer are enough.
■ **PAUSE POINT** The eluate can be stored at 4 °C overnight without loss of nuclease activity.
(xv) Analyze the purity of the Cas9 protein by SDS-PAGE. Mix 15 µl of eluate with 3 µl of 6× loading buffer, boil at 95 °C for 10 min and centrifuge at 12,000*g* for 1 min at room temperature. Load the sample onto a 10% (wt/vol) SDS-PAGE gel and run at 120 V for 50 min.
(xvi) Using a 30-kDa MWCO concentration column, exchange the buffer of the purified protein with Cas9 storage buffer. Determine the final concentration of Cas9 protein with the Bradford assay[47]; a concentration of 1–2 mg/ml is suitable.
▲ **CRITICAL STEP** Buffer D is an alternative buffer for Cas9 storage that also works well in our laboratory.
(xvii) Make 30- to 50-µl aliquots of the Cas9 protein in RNase-free 1.5-ml tubes for single use. Quick-freeze in liquid nitrogen and store at −80 °C.
▲ **CRITICAL STEP** Avoid repeated freezing–thawing of Cas9 protein; discard aliquots once thawed.
■ **PAUSE POINT** Cas9 protein can be stored at −80 °C for at least 1 year.

Assessment of the *in vitro* cleavage activity of CRISPR/Cas9 RNPs ● TIMING 1 d
▲ **CRITICAL** Use RNase-free microcentrifuge tubes and tips for Steps 15–19.

15| PCR amplify fragments containing the target site from genomic DNA using target-specific primers (**Table 1**) and run on an agarose gel as described in Steps 2–4.

16| Purify the amplicons with an EasyPure PCR purification kit, following the manufacturer's instructions. Add 30 µl of RNase-free water for elution.

17| Prepare the following reaction mix to detect Cas9 nuclease activity. Mix thoroughly and incubate at 37 °C for 1 h.

Component	Amount (µl)
Cas9 protein (from Step 14B(xvii))	0.5 (1 µg)
sgRNA (from Step 13)	0.5 (1 µg)
10× Cas9 reaction buffer	2
PCR amplicons (from Step 16)	× (100 ng)
RNase-free water	Up to 20

18| Heat at 65 °C for 10 min and add 3 µl of DNA loading buffer. Run on a 2% (wt/vol) agarose gel at 120 V for 20 min.
▲ **CRITICAL STEP** Mixtures that are not preheated may remain blocked in the agarose gel and not move.

19| Analyze the cleavage activity by quantifying the DNA bands using gel quantification software (ImageJ). Usually, at least >70% cleavage activity is expected[40].
? TROUBLESHOOTING

***In vivo* validation of CRISPR/Cas9 RNPs using transient protoplast assays ● TIMING 5 d**
▲ **CRITICAL** The plasmids encoding sgRNA and Cas9 in Step 1 can be used as a positive control.

20| Isolate wheat protoplasts using detailed protocols found in our previous published methods[38].
▲ **CRITICAL STEP** Prepare the cell-wall-dissolving enzyme solution with RNase-free water for RNP transfection.
▲ **CRITICAL STEP** Prepare the W5 solution with ddH_2O and autoclave at 121 °C for 20 min for RNP transfection.

21| *PEG-mediated transfection*. Add 10–20 µl of Cas9 protein (20 µg, from Step 14B(xvii)) and 10 µl of sgRNA (20 µg, from Step 13) to an autoclaved 2-ml microcentrifuge tube.

22| Add 200 µl of protoplasts (5×10^5 cells from Step 20) and mix gently.

23| Add 240 µl of freshly-prepared PEG solution, and mix thoroughly by gently tapping the tube.
▲ **CRITICAL STEP** Prepare fresh PEG solution with RNase-free water.

24| Incubate the mixture in the dark for 20 min.

25| Add 900 µl of W5 solution to the tube and mix by gently inverting to stop transfection.

26| Centrifuge at 100*g* for 3 min at room temperature with slow acceleration and braking.
▲ **CRITICAL STEP** The Eppendorf 5810r centrifuge provides adjustment to reach the highest speed with different acceleration speeds. Setting the acceleration and braking at 3 is a gentle level, which can avoid breaking of the protoplast cells

27| Remove the supernatant and resuspend the pellet in 1 ml of W5 solution.

28| Incubate the mixture in the microcentrifuge tube in the dark at 23 °C for 2 d.
▲ **CRITICAL STEP** Place the microcentrifuge tube in a horizontal position to maintain the protoplasts in a better state.

29| Collect the protoplasts 2-d post transfection by centrifuging at 12,000*g* for 2 min, at room temperature, and then discarding the supernatant. Extract genomic DNA by the cetyltrimethyl ammonium bromide (CTAB) method[48].

30| Detect CRISPR/Cas9 RNP nuclease activity in the protoplasts by the PCR/RE or T7EI assay as described in our previous protocol[38] (**Table 1**).
? TROUBLESHOOTING

Growth of wheat plants ● TIMING 90 d
31| Grow single wheat plants (cultivar Kenong199) in individual 190-mm pots in a greenhouse. For germination, maintain daytime temperatures at 18–20 °C and nighttime temperatures at 10–12 °C for 7–10 d. Move the seedlings to a 4 °C cold house for 3 w for vernalization. Return to the greenhouse for 3 weeks. Raise the daytime temperature to 28 °C and the nighttime temperature to 18 °C for the jointing and earing stages. Maintain the light on the long-day photoperiod at a light/dark (LD) ratio of 16:8 during the entire procedure.
▲ **CRITICAL STEP** The quality of the immature embryos is very important for transformation. Air-conditioning and supplementary lights are needed to maintain a suitable environment for growth.

Harvesting of immature embryos and biolistic delivery of CRISPR/Cas9 IVTs or RNPs ● TIMING 1–2 d
32| Harvest the ears at 12–14 d after pollination (DAP).
▲ **CRITICAL STEP** The right stage for harvesting ears depends on the genotype and environment. Immature embryos of ~1 mm in length are at the right developmental stage. Usually each plantlet contains 7–8 tillers, each of which yields 15–20 effective immature embryos.
■ **PAUSE POINT** The harvested ears can be dipped into 75% (vol/vol) ethanol for 1 min and washed two times with water for sterilization, and then can be wrapped in wet paper and stored at 4 °C for up to 5 d.

33| Detach the kernels from the ears at room temperature.
■ **PAUSE POINT** The detached kernels can be stored at 4 °C overnight.

34| Wash the kernels with 75% (vol/vol) ethanol for 1 min. Discard the ethanol and wash with 2.5% (wt/vol) sodium hypochlorite (NaClO) for 20 min. Then wash six times with autoclaved ddH_2O.

35| Using a sharp blade, collect the immature embryos under an optical microscope. Place ~80 embryos into high-osmotic medium in each Petri dish.
▲ **CRITICAL STEP** Complete this step within 1 h. A skilled technician can obtain ~300 embryos.
▲ **CRITICAL STEP** Keep the epiblast in contact with the medium, with the scutellum upward.

36| Incubate the embryos in the high-osmotic medium for 3–4 h before bombardment.
▲ **CRITICAL** Use RNase-free microfuges and tips.

37| Use option A for the biolistic delivery of CRISPR/Cas9 IVTs or option B for the biolistic delivery of RNPs.

(A) Biolistic delivery of CRISPR/Cas9 IVTs

(i) Assemble the following mixture for ten shots of RNA coating: Incubate the mixture at −20 °C for at least 1 h.

Component	Amount (μl)
Gold nanoparticles (40 mg/ml)	50
Cas9 mRNA (from Step 14A(vii))	20 (10 μg)
sgRNA (from Step 13)	10 (10 μg)
Ammonium acetate (5 M)	10
2-propanol	200

■ **PAUSE POINT** Incubation for 3–4 h also works well.

(ii) Centrifuge the sample at 10,000*g* for 10 s at room temperature and discard the supernatant.

(iii) Resuspend the pellet in 1 ml of absolute ethanol with pipetting.

(iv) Centrifuge at 10,000*g* for 10 s at room temperature and discard the supernatant. Resuspend in 200 μl of absolute ethanol (20 μl for each shot).
▲ **CRITICAL STEP** Mix the suspension thoroughly by pipetting.

(v) Using a pipette, slowly spread 20 μl of the suspension onto the central region of a macro-carrier previously positioned in the membrane support, which is part of the bombardment system. Air-dry at room temperature for 5 min.
▲ **CRITICAL STEP** Spread slowly to prevent outflow of the central region.

(vi) Perform biolistic transformation of embryos from Step 36 using a PDS1000/He particle bombardment system with a target distance (between target cell and stopping screen) of 6.0 cm at a helium pressure of 1,100 p.s.i. ~80 embryos are used per shot.
▲ **CRITICAL STEP** Perform the transformation as soon as possible after drying.

(vii) Incubate the bombarded embryos in high-osmotic medium overnight in the dark at 23 °C.

(B) Biolistic delivery of CRISPR/Cas9 RNPs

(i) Set up the following mixture to form sufficient RNPs for ten shots: Incubate at 25 °C for 10 min.

Component	Amount (μl)
Cas9 protein (from Step 14B(xvii))	10 (20 μg)
sgRNA (from Step 13)	10 (20 μg)
10× Cas9 reaction buffer	10
RNase-free water	70

(ii) Add 50 µl of gold nanoparticles to the mixture. Mix gently and thoroughly by pipetting.
(iii) Spread 15 µl of the mixture onto the central region of each macro-carrier. Air-dry on the benchtop at room temperature.
▲ CRITICAL STEP Too much glycerol in the Cas9 storage and reaction buffer may result in failure to air-dry for coating.
? TROUBLESHOOTING
■ PAUSE POINT Air-drying usually takes 1–2 h.
(iv) Perform the particle bombardment and incubation as described in Step 37A(vi and vii).

Verification of delivery by deep sequencing ● TIMING 3–4 weeks

38| Collect 100 bombarded embryos at random in a microcentrifuge tube 2 d after transformation.

39| Extract genomic DNA by the CTAB method[48].

40| Using homolog-specific primer sets, perform a first round of PCR to amplify the regions containing the target sites (**Table 1**), and run the products on an agarose gel as described in Steps 2–4 (**Fig. 2**).

41| Purify the amplicons with an EasyPure PCR purification kit, following the manufacturer's instructions. Add 30 µl of ddH_2O for elution. The concentration is usually ~20–40 ng/µl.

42| Perform the second round of PCR to add barcodes to the amplicons by incorporating barcode sequences into the primers (**Fig. 2**; **Table 1**). Using the purified products from the first round of PCR (Step 41), set up and run the PCR reaction as described in Steps 2 and 3.

43| Run all 50-µl PCR products on a 1.2% (wt/vol) agarose gel in TAE buffer at 120 V for 15–20 min.

44| Cut off the gel with the destination band (single band of ~110–130 bp is expected) and purify with an AxyPrep DNA Gel Extraction Kit according to the manufacturer's instructions. Concentrations may vary from 10 to 30 ng/µl.

45| Pool equal amounts of each PCR product (200 ng per sample). Send the pool to a commercial company for library construction and sequencing.

46| To measure the mutation frequency, analyze the sequence data with Cas-Analyzer (http://www.rgenome.net/cas-analyzer/#!) or CRISPResso (http://crispresso.rocks/), or have it analyzed by a bioinformatics technician. Successful detection of targeted mutagenesis indicates that the RNPs have been delivered into the explants.

Tissue culture to regenerate testable seedlings ● TIMING 6–8 weeks

▲ CRITICAL No antibiotic is used for selection in the entire tissue culture procedure.

47| Transfer the bombarded embryos (from Step 37) to recovery medium and incubate at 23 °C for 14 d in the dark.
▲ CRITICAL STEP Place 30 embryos in each plate, and calli of ~4–6 mm will be formed.

48| Transfer the calli to regeneration medium at 23 °C for 14 d with a day photoperiod of LD 16:8.
▲ CRITICAL STEP Green tips will form on the surface of the calli. Usually more than half of the calli have the ability to differentiate green tips.

49| Detach the green tips from the calli with forceps and separate them into two to three small pieces. Transfer the green tips to new regeneration medium and incubate at 23 °C for 14 d with a day photoperiod of LD 16:8. For most bombarded embryos, 2–4 plantlets can be regenerated.

50| Transfer the regenerated plantlets to rooting medium and incubate at 23 °C for 7–10 d with a day photoperiod of LD 16:8.
▲ CRITICAL STEP Seedlings of ~5–6 cm in the Petri dishes are ready for screening for mutants.
? TROUBLESHOOTING

Mutant screening and identification ● **TIMING 7–10 d**

51| Aseptically cut off a piece of leaf (~10 × 3 mm) from each of 3–4 plantlets (usually regenerated from the same bombarded embryo) and pool the leaf pieces.

52| Extract genomic DNA using the DNA Quick Plant System, following the manufacturer's instructions. Add 50 µl of ddH_2O for elution; the final concentration will vary from 100 to 200 ng/µl.

53| PCR-amplify the targeted regions with conserved primers that can recognize the three homologs simultaneously (**Table 1**). Assemble the reaction as follows:

Component	Amount (µl)
Genomic DNA (from Step 52)	1
2× Taq MasterMix	10
Conserved primer-F	1
Conserved primer-R	1
ddH_2O	7

54| Perform PCR reactions using the following conditions:

Cycle number	Denaturation	Annealing	Extension
1	94 °C, 3 min		
2–36	94 °C, 30 s	Tm °C, 30 s	72 °C, 30 s
37			72 °C, 2 min

Tm, melting temperature.

55| Run 5 µl of the PCR product on a 1.2% (wt/vol) agarose gel in TAE buffer at 140 V for 15 min. A single band is expected.

56| Digest the PCR products with the appropriate restriction enzyme according to the manufacturer's instructions. Assemble the reaction as follows:

Component	Amount (µl)
PCR products (from Step 54)	3
10× Fastdigest buffer	1
Restriction enzymes	0.3
ddH_2O	6.7

▲ **CRITICAL STEP** More of the PCR products can be used for digestion, depending on the concentration of the PCR products.

57| Run all 10 µl of digested products on a 2.0% (wt/vol) agarose gel in TAE buffer at 140 V for 15–20 min. Usually, three to four mutants can be identified in each 96-well plate.
? TROUBLESHOOTING

58| Transfer those plantlets in the pool that give positive signals (samples that contain uncut bands after restriction enzyme digestion) to new rooting medium and grow for 5–7 d.
▲ **CRITICAL STEP** This step can be eliminated if the seedlings are large enough for sampling.

59| Sample the individual plantlets from Step 46. Screen for mutants by PCR/RE assays as described in Steps 52–57 using a conserved primer set (**Table 1**).

60| Amplify the detected mutants with homolog-specific primer sets (**Table 1**).

61| Identify the mutation types by PCR/RE as described in Steps 53–57 and confirm by sequencing.
? TROUBLESHOOTING

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

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	No highly active sgRNA	sgRNA activity may depend on the genomic context	Design more sgRNAs and detect their efficiencies in protoplast assays
13	Pellet cannot be completely dissolved	Contamination with impurities such as T7 RNA polymerase	After adding RNase-free H_2O, the sgRNA is dissolved in the supernatant. The remaining pellet may have impurities these can be removed by centrifuging or purifying the ssDNA/RNA using a GeneJET RNA Cleanup and Concentration Micro Kit
19	Poor Cas9 cleavage activity	Contamination and protein misfolding	Purify Cas9 at 4 °C or on ice
30	No activity was detected in protoplast assays	Low transfection efficiency of the protoplasts	Use a GFP-positive plasmid as a positive control and expect a >50% transfection efficiency. Use the GW2-sgRNA as a positive control
37B(iii)	RNPs failed to air-dry after 2–3 h or were sticky on the macro-carrier after biolistic delivery	Too much glycerol in the mixture	Use the buffer strictly as shown in our protocols
50	Limited number of testable plantlets	Low regeneration efficiency of embryos	Check the conditions for plant growth and the stage of immature embryos
57	Incomplete digestion of all samples, including negative control	Incorrect restriction conditions; too many PCR products	Check that optimum buffer and temperature were used; use more enzymes and digest fewer PCR products for a longer time
	Few, if any mutant plants	Insufficient delivery of the CRISPR/Cas9 reagents	Confirm delivery and nuclease activity by deep sequencing
61	SNP away from the target sites	Polymerase-induced errors	Use a high-fidelity DNA polymerase such as FastPfu DNA polymerase to confirm mutation types

● TIMING
Step 1, sgRNA design, cloning and validation: ~10 d
Steps 2–13, *in vitro* transcription of sgRNA: 1–2 d
Step 14A, *in vitro* transcription of Cas9 mRNA: 1 d
Step 14B, expression and purification of Cas9 protein from bacteria: 3–4 d
Steps 15–19, testing CRISPR/Cas9 RNP nuclease activity by *in vitro* cleavage: 1 d
Steps 20–30, confirmation of nuclease activity of CRISPR/Cas9 RNPs using protoplast assays: 5 d
Step 31, growth of wheat plants (should be prepared in advance): 90 d
Steps 32–37, harvesting of immature embryos and biolistic delivery of CRISPR/Cas9 IVTs or RNPs: 1–2 d

Steps 38–46, confirming biolistic delivery by deep sequencing: 3–4 weeks
Steps 47–50, regeneration of testable plantlets: 6–8 weeks
Steps 51–61, screening of mutants and confirmation of sequences: 7–10 d

ANTICIPATED RESULTS

On the basis of this protocol, we have obtained DNA-free genome-edited mutants via transient activity of CRISPR/Cas9 IVTs/RNPs. We designed sgRNAs to target three genes, *GASR7*, *LOX2* and *CER9* (**Fig. 3a**). All the sgRNAs were validated in protoplasts using our previous protocol[38]. Using CRISPR/Cas9 IVTs, we obtained a 1.8% MPE (mutant production efficiency = number of mutants/number of bombarded embryos) for *GASR7* in the T0 generation in a wheat variety (Kenong 199) (**Fig. 3b**). The *in vitro* cleavage assays demonstrated that bacterially expressed Cas9 protein, along with purified sgRNAs, resulted in robust cleavage activity (**Fig. 3c**). We also demonstrated CRISPR/Cas9 delivery in the form of RNPs in protoplasts, and obtained high mutation frequencies for several target genes (**Fig. 3d**). gasr7-RNPs were successfully used in a different wheat variety (YZ814) with the MPE being 1.8%, suggesting that this method is more likely to be applied in other genotypes (**Fig. 3e**).

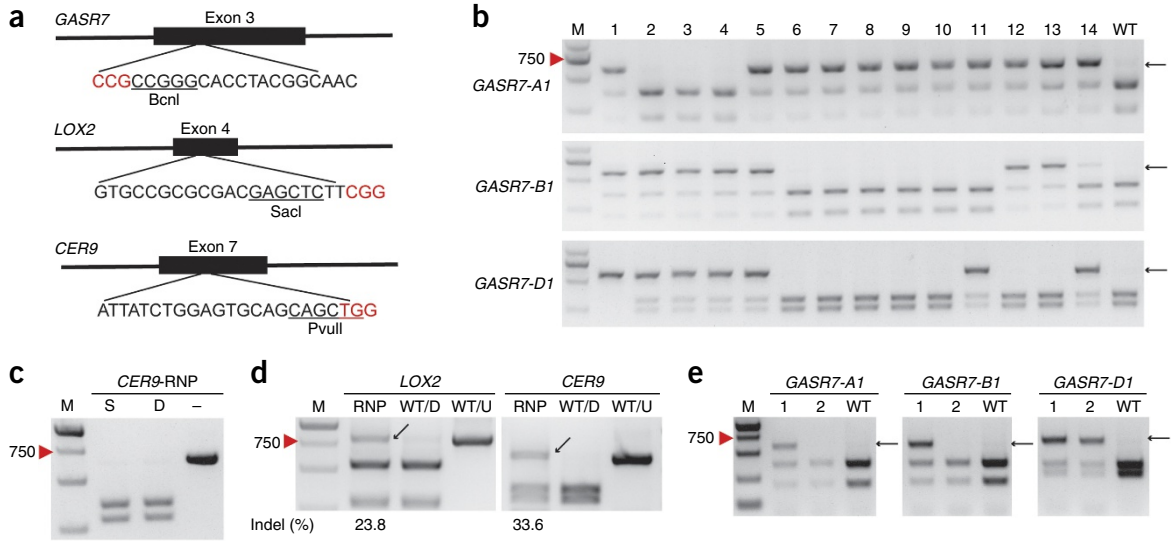

Figure 3 | Anticipated results for mutagenesis by CRISPR/Cas IVTs/RNPs. (**a**) Three sgRNAs were designed to target *GASR7*, *LOX2* and *CER9*, respectively (**Supplementary Table 1**). PAM sequences are highlighted in red and the corresponding restriction enzymes are underlined. (**b**) Agarose gel showing mutations mediated by gasr7-IVTs in the T0 generation detected by PCR/RE assays using homolog-specific primers (**Table 1**) (Step 61). Non-cleaved mutant bands are indicated by black arrows. (**c**) *In vitro* cleavage assay of cer9-RNPs at the cer9 locus detected with conserved primers (Step 18) comparing two storage buffers. Both work well in our lab, and readers can choose which to use. 'S' indicates Cas9 stored in Cas9 storage buffer, 'D' indicates Cas9 in buffer D and '–' indicates the negative control. (**d**) Validation of nuclease activities of lox2-RNPs and cer9-RNPs using transient protoplast assays detected by PCR/RE assays. Black arrows indicate uncleaved mutant bands. Mutagenesis efficiencies are calculated by dividing the density of uncleaved bands by the density of all DNA bands in the same lane. Lanes labeled with 'WT/D' and 'WT/U' are the PCR products amplified from wild-type (WT) plants with and without restriction enzyme digestion, respectively. (**e**) Agarose gel showing mutations in the T0 generation of wheat variety (YZ814) caused by gasr7-RNPs and detected by PCR/RE assays with homolog-specific primers (**Table 1**) (Step 61). Non-cleaved mutant bands are indicated by black arrows.

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

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