Establishment of an efficient seed fluorescence reporter-assisted CRISPR/Cas9 gene editing in maize

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ABSTRACT

Genome editing by clustered regularly interspaced short palindromic sequences (CRISPR)/CRISPR-associated protein 9 (Cas9) has revolutionized functional gene analysis and genetic improvement. While reporter-assisted CRISPR/Cas systems can greatly facilitate the selection of genome-edited plants produced via stable transformation, this approach has not been well established in seed crops. Here, we established the seed fluorescence reporter (SFR)-assisted CRISPR/Cas9 systems in maize (Zea mays L.), using the red fluorescent DsRED protein expressed in the endosperm (En-SFR/Cas9), embryos (Em-SFR/Cas9), or both tissues (Em/En-SFR/Cas9). All three SFRs showed distinct fluorescent patterns in the seed endosperm and embryo that allowed the selection of seeds carrying the transgene of having segregated the transgene out. We describe several case studies of the implementation of En-SFR/Cas9, Em-SFR/Cas9, and Em/En-SFR/Cas9 to identify plants not harboring the genome-editing cassette but carrying the desired mutations at target genes in single genes or in small-scale mutant libraries, and report on the successful generation of single-target mutants and/or mutant libraries with En-SFR/Cas9, Em-SFR/Cas9, and Em/En-SFR/Cas9. SFR-assisted genome editing may have particular value for application scenarios with a low transformation frequency and may be extended to other important monocot seed crops.

Keywords: CRISPR/Cas9, embryo-specific reporter, endosperm-specific reporter, genome editing, maize, seed fluorescence reporter

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INTRODUCTION

Genome editing via clustered regularly interspaced short palindromic sequences (CRISPR) and the CRISPR-associated protein (Cas) is revolutionizing our approach to generating a wide range of specific, targeted mutations in many species. The introduction of desired mutations in the promoter or coding sequences of genes of interest have brought genome-editing technologies to the forefront as powerful tools that enable both the study of gene function and crop improvement (Zhang et al., 2017; Chen et al., 2019). Due to its precision to the nucleotide level, genome editing has become the main approach for breeding-by-design in agriculture, showing its unparalleled technical advantages (Xie et al., 2020). Indeed, genome editing has already been applied to manipulating important crop traits such as grain

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yield and quality, abiotic stress resistance, male sterility, and virus resistance, with immense added agricultural value (Chen et al., 2019 and many references in the citations). In addition, genome editing technologies now offer realistic solutions for the long-standing needs of agriculture. For example, mutations in multiple genes (multiplex mutations) can now be successfully produced at once; genome editing also opened the possibility of asexual reproduction for the clonal maintenance of hybrids through seed propagation (Khanday et al., 2019; Wang et al., 2019a). As many crops are sown as hybrids to harness hybrid vigor, male-sterile lines have been widely employed during seed production; genome editing now makes it possible to efficiently and easily sort sterile line and maintainer line seeds in maize (Zea mays), as we previously reported (Qi et al., 2020b).

The CRISPR/Cas9 system uses two components: the single guide RNA (sgRNA) and the Cas9 nuclease (Jinek et al., 2012). CRISPR/Cas9 was first applied to the editing of plant genes in 2013 (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013) and has since become the prevalent gene editing tool for both fundamental studies and crop improvement. Currently, most targeted mutations are generated through the stable introduction of a CRISPR/Cas expression cassette in plants to generate single (Zhao et al., 2016; Li et al., 2017, 2019), multiplex (Ma et al., 2015), or mutant libraries (Lu et al., 2017b; Meng et al., 2017; Bai et al., 2020; Liu et al., 2020). This method requires CRISPR/Cas activity in vivo to generate the targeted mutation(s) at the desired site(s), followed by the selection of transgene-free plants to remove the CRISPR/Cas expression cassette (Dong et al., 2019). Since the exogenous CRISPR/Cas components are no longer present, the mutants selected do not suffer the effects of continuous editing activity. In addition, the removal of the transgene can alleviate consumer concerns regarding the use of transgenic plants in agriculture (He et al., 2018). Therefore, the presence or absence of the transgene should be verified or screened for via genotyping or other detection methods, which can be laborious and time-consuming.

To solve this problem, several studies have turned to reporter or selection systems. For example, the Arabidopsis (Arabidopsis thaliana) PRODUCTION OF ANTHOCYANIN PIGMENTS 1 (PAP1) gene was included with the CRISPR/Cas9 vector to serve as a visual marker for the presence/absence of the construct in tobacco (Nicotiana tabacum) by inducing the strong accumulation of purple anthocyanin pigments in transgenic leaves (Liu et al., 2019). However, this selection system is not always reliable, due to the complex regulatory networks of some monocot species; in addition, anthocyanin biosynthesis has been reported to be a poor selection method in some germplasms due to complete marker inhibition in maize (Chaikam et al., 2015). Another developed strategy is CRISPR-S, in which an RNA interference cassette targeting a cytochrome P450 gene the silencing of which confers herbicide sensitivity in rice (Oryza sativa) (Lu et al., 2017a). However, this strategy still requires genotyping to determine the presence of the transgene and herbicide treatment when necessary. Another system in rice consisted of a self-elimination CRISPR/Cas9 vector driving the expression of a toxin in the embryo and a cytoplasmic male sterility gene resulting in male gametophyte death; this suicide transgene allows the self-elimination of plants carrying the original transgene (He et al., 2018). This system was designed to obtain transgene-free mutants; however, other technologies necessitate that the transgene remain in transformants as alternative technological components, such as for in vivo targeted mutations during backcrossing (Li et al., 2017; Qi et al., 2020a) or during haploid inductions (Keliiher et al., 2019; Wang et al., 2019b). Another ideal system relies on a visible marker system in the seeds of seed plants. For instance, an mCherry fluorescence marker in Arabidopsis seeds has been implemented as a proxy for the presence of the transgene, resulting in a reduction of the workload by over 75% (Gao et al., 2016). However, this system also exhibited some genetic stability issues in Arabidopsis (He et al., 2018).

We previously developed maize seed endosperm- and/or embryo-specific fluorescent reporters for haploid kernel identification (Dong et al., 2018) as well as reporters for sorting seeds from genic male-sterile (GMS) lines and maintainer lines (Qi et al., 2020a). These endosperm- and embryo-specific fluorescent reporters were also successfully deployed in other monocot species, including rice, wheat (Triticum aestivum), and barley (Hordeum vulgare) (Dong et al., 2018), thus showing clear promise for the development of a reliable seed fluorescence reporter (SFR)-assisted CRISPR-Cas9 system (SFR/Cas9). We report here the development and implementation of three SFR/Cas9 systems, which incorporate the gene encoding the red fluorescent protein DsRed2, driven by endosperm- and/or embryo-specific promoters to allow easy and efficient seed sorting. We further demonstrate the usefulness of our strategy to assist in the selection of transgene-free genome-edited plants at one or multiple sites. The strategy described here will further improve the efficiency of subpooling possibilities by offering two tissue-specific reporters, and may be applicable to other monocot seed crops.

RESULTS

Rationale of SFR design and generation of SFR/Cas9 maize plants

To establish visible seed reporter systems in maize, we constructed an SFR expression cassette consisting of the embryo-specific promoter (named ZmESP) of Zm3996 gene (Liu et al., 2014) or the endosperm alleurone-specific promoter (named HvASP), from the barley Lipid Transfer Protein2 (Ltp2) gene (Kalla et al., 1994) within the CRISPR/Cas9 expression cassette (Figure 1A, B). We will refer to the endosperm-specific SFR as En-SFR/Cas9, and the embryo-specific SFR as Em-SFR/Cas9. These two tissue-specific SFR systems are more advantageous during mutant library generation over single seed-wide fluorescent reporter systems, while also offering the possibility of sorting seeds across each tissue into subpools when the
identification of a narrower scope of target mutants is needed (Figure 1C).

To test the efficiency of these SFR/Cas9 systems, we transformed immature maize embryos with 14 En-SFR/Cas9 and/or Em-SFR/Cas9 plasmids, each targeting a distinct gene (Tables S1, S2) using Agrobacterium-mediated transformation. Transformations were carried out in three independent transformation experiments: one with En0-SFR/Cas9 targeting one locus, one with Em0-SFR/Cas9 targeting another locus, and one pool of 12 plasmids (six from En0-SFR/Cas9 and six from Em0-SFR/Cas9). We obtained 38 primary transformants for En0-SFR/Cas9 (Table S3), 18 transformants for Em0-SFR/Cas9 (Table S4), and 81 transformants from the pooled transformation (Table S5).

Characterization of SFR/Cas9 in maize ears, kernels and cells

To evaluate the usefulness of our engineered SFR as a seed reporter, we scored fluorescence in maize ears (Figure 2A), kernels (Figure 2C), longitudinal section of kernels (Figure 2B, D), and by confocal microscopy (Figure 2E) from Em-SFR/Cas9, En-SFR/Cas9, and Em/En-SFR/Cas9 transformants. In maize ears, individual fluorescent kernels harboring the En-SFR transgene were easily distinguished (white arrowhead in Figure 2A), compared to non-fluorescent kernels, which presumably do not carry the transgene. Similarly, red fluorescence was clearly detectable from longitudinal kernel sections, as illustrated in Figure 2B for an Em-SFR seed. We will note here that red fluorescence from Em-SFR seeds was readily visible when seeds and longitudinal sections were observed under bright white light (Figure 2C, D).

However, we expect that seed sorting will be greatly facilitated under fluorescent protein specifically excited at 550 nm with an inspection light equipped with the proper excitation wavelength filter. As shown in Figure 2C-E, kernels harboring both Em-SFR and En-SFR were easily identified on the basis of their kernel-wide red fluorescence. Conversely, kernels carrying only En-SFR exhibited no red fluorescence in the embryo, while individual kernels harboring Em-SFR lacked fluorescence around the fluorescent embryo (Figure 2C). To visualize the fluorescence conferred by SFR systems at the cellular level, we next examined kernel section by confocal microscopy observations for all SFR systems (Figure 2E). We detected red fluorescence in seeds carrying En-SFR within the aleurone layer, while kernels harboring Em-SFR showed fluorescence in the cells of the embryo. Kernels carrying both systems (En/En-SFR) displayed fluorescence in both the aleurone layer of the endosperm and the embryo. These observations are consistent with our previous reports using the same promoters (Dong et al., 2018). Next, to determine how often red fluorescence correlated with the presence of the transgene in the progeny of primary transformants, we genotyped 16 T1 plants of each for SpCas9 and used Bar strip tests (Figure S1). Both tests demonstrated that the fluorescence status of a kernel acts as a reliable visible reporter for sorting kernels based on the presence/absence of the transgene.

Case study using a single En-SFR/Cas9

To evaluate the efficiency of our system with a single En-SFR, we analyzed transformants of the E0-SFR/Cas9 construct, which targeted Indeterminate gametophyte 1 (ZmIg1; Table S1). We obtained 38 primary T0 transformants that set seeds after
self-pollination or crossing with wild-type plants (Table S3). We photographed one example maize ear (from Event #IG182) under bright field illumination and fluorescence excitation (Figure 3A) to illustrate the segregation of the transgene. We selected dark kernels without fluorescence, indicative of the absence of the En0-SFR/Cas9 cassette (Figure 3A right panel) and planted them to generate material for genotyping and set T₁ seeds. We sequenced the target site near the ZmIg1 start codon to assess editing status (Figure 3B), resulting in the identification of 14 transgene-free T₁ lines, derived from two T₀ plants analyzed, and carrying a mutant allele within the target region (Figure 3C; Table S3). We confirmed the loss of the transgene in each line with the Bar strip test and by polymerase chain reaction (PCR) genotyping for the presence of SpCas9. These results therefore indicated that the En-SFR/Cas9 system works well for genome editing of single targets.

Case study using a single Em-SFR/Cas9

Next, we tested the efficiency of genome editing with a single Em-SFR construct that was designed to target

Figure 2. Characterization of seed fluorescence reporter (SFR)/clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) in ears, kernels and cells

(A) A typical En-SFR/Cas9 maize ear (Event #IG178; Table S3) under brightfield (left) and upon excitation of red fluorescence (right) using an inspection light equipped with a 550 nm excitation wavelength/filter. The white arrows point to the same kernel in the two photographs. Scale bars, 1 cm. (B) Longitudinal section of a typical Em-SFR/Cas9 maize kernel (Event#: PT232; Table S4) showing tissue-specific red fluorescence. Em, embryo, En, endosperm. Scale bars, 2 mm. (C–E) Characterization of SFRs in maize whole kernels (C), longitudinal sections (D), and confocal microscopy (E). Em/En (Event #125; Table S5), kernels harboring both Em- and En-SFRs. In (D) and (E), the white arrowheads indicate aleurone cells forming a single layer around the embryo. Scale bars, 2 mm (C, D) or 100 µm (E).
Zm00001d024337 (Table S1). We analyzed 18 primary transformants with harvested ears (Table S4). We photographed one example maize ear, in which some kernels were removed to expose the embryo from other kernels, under bright field illumination and fluorescence excitation (Figure 4A). As described above with the single En-SFR construct, we retained dark kernels without fluorescence indicative of the loss of the Em0-SFR/Cas9 transgene and planted for genotyping and seed setting. We then sequenced the target site, which was designed to edit the genomic region near the start codon of Zm00001d024337 (Figure 4B). We obtained 10 transgene-free T1 lines carrying a mutant allele within the target region from two T0 events (Figure 4C; Table S4). We validated the absence of the transgene with Bar strip tests and PCR genotyping for the presence

Figure 3. An example of endosperm-seed fluorescence reporter/clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (En-SFR/Cas9) designed to target maize Indeterminate gametophyte 1 (ZmIg1) (A) Images of the same maize ear (Event #IG182) carrying En-SFR/Cas9 under brightfield (left panel) and upon excitation of red fluorescence (right) using an inspection light equipped with a 550 nm excitation wavelength/filter. Scale bars = 1 cm. (B) Schematic representation of the target site in the ZmIg1 gene (Gene ID: Zm00001d042560). The red arrow indicates the target site. Black boxes indicate exons, white boxes indicate untranslated regions. (C) Examples of mutations detected at the ZmIg1 target site. The underlined sequence indicates the protospacer adjacent motif (PAM). Sequences in bold blue indicate the single guide RNA-associated region. Sequences in red are insertions or substitutions. Each event shows the genotype at both copies of the target site.

Figure 4. An example of embryo-seed fluorescence reporter/clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Em-SFR/Cas9) designed to target Zm00001d024337 (A) Images of the same maize ear (from Event #PT434) carrying Em-SFR/Cas9 under brightfield (left panel) and upon excitation of red fluorescence (right) using an inspection light equipped with a 550 nm excitation wavelength/filter. Scale bars = 1 cm. The white arrows point to the same kernels in both panels. (B) Schematic representation of the target site in Zm00001d024337. The red arrow indicates the target site. Black boxes indicate exons, white boxes indicate untranslated regions. (C) Examples of mutations detected at the Zm00001d024337 target site. The underlined sequence indicates the protospacer adjacent motif (PAM). Sequences in bold blue indicate the single guide RNA-associated region. Sequences in red are insertions or substitutions. Each event shows the genotype at both copies of the target site.
Seed sorting accelerated genome editing of SpCas9. These data supported the efficient isolation of genome-edited plants from a single construct with the Em-SFR/Cas9 system.

**Generation of small-scale mutant libraries using pooled En- and Em-SFR/Cas9 constructs**

Seed fluorescence reporter/Cas9 systems should also be amenable to the generation of mutant libraries using a small- or large-scale pooling strategy. In addition, using two tissue-specific SFRs such as En-SFR/Cas9 and Em-SFR/Cas9 may be advantageous as they may reduce the workload of target identification through subpools sorted based on the fluorescence pattern, when distinct sgRNAs are combined with either En- and Em-based systems. To test this hypothesis, we performed a single transformation with pools of six Em-SFR/Cas9 combined with six Em-SFR/Cas9 constructs, each targeting a different gene (Table S1), from which we obtained 81 T₀ primary transformants that set seeds (Table S5). The ear of Event #125 is shown as an example of Em/En-SFR, as well as individual kernels carrying Em-, En- and Em/En-SFRs (Figure 5A). Of 81 events, 44 harbored an Em-SFR transgene, as determined by the endosperm-specific fluorescence pattern of kernels. Another 23 events carried an Em-SFR transgene, and the final 12 events appeared to harbor both Em-SFR and En-SFR transgenes. Two T₀ primary transformants exhibited no fluorescence in either tissue. The ears were then divided into En, Em, and Em/En subpools (Figures 1C, 5B). All seeds exhibiting red fluorescence were planted and allowed to set T₁ seeds. We then determined which SFR/Cas9 vector each T₁ individuals carried by nested PCR and sequenced the genomic region targeted by the respective constructs. Each SFR-positive event resulted in the identification of single or multiplex mutants depending on the T₁ plant analyzed (Table S5). All mutant types identified among the En, Em, and Em/En subpools are provided in Tables 1 and Table S6. We successfully identified single genome-edited mutants for each targeted gene from both En and Em subpools. In addition, we identified four sets of double mutants among the En and Em subpools, and six sets of double mutants and four sets of...

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**Figure 5.** An example of pooled seed fluorescence reporter (SFR)/clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) systems designed to target 12 separate genes (A) Images of the same maize ear carrying SFR-Em/En under brightfield (left panel) and upon excitation of red fluorescence (right) using an inspection light equipped with a 550 nm excitation wavelength/filter. Scale bars = 1 cm. All possible fluorescence patterns from SFR systems in single kernels are shown below the ear: WT (no fluorescence), Em/En (fluorescence in both embryo and endosperm), Em (fluorescence only in embryo), and En (fluorescence only in endosperm). (B) Pooling strategy to identify the target mutant and/or multiplex mutants from subpools, after sorting based on seed fluorescence pattern. Em1-6 and En1-6 refer to the constructs designed to target each selected gene (Table S1). (C) Example of genotypes from five individuals from a single transformation event harboring mutations in both Zm00001d040611 and Zm00001d033267. The underlined sequence indicates the protospacer adjacent motif (PAM). Sequences in bold blue indicate the single guide RNA-associated region. Sequences in red are insertions or substitutions. Each event shows the genotype at both copies of the target site.
of triple mutants among the Em/En subpool. In summary, we identified 12 single mutants, 13 double mutants and four triple mutants among 79 SFR/Cas9 transformants (Tables S5, Table S6).

DISCUSSION

Promising and indispensable application scenarios for SFR

Our data and observations indicate that both En- and Em-SFR systems are effective reporters for both the presence and absence of a transgene, thereby reducing the workload associated with selection and improving work efficiency. We anticipate that the SFR system will be an important technical component that will support the implementation of certain applications. For example, the one-step genome editing of elite varieties was reported using maternal haploid induction (Kelliher et al., 2019; Wang et al., 2019b), but the frequency of the HI-Edit method (Kelliher et al., 2019) or haploid-inducer mediated genome editing (IMGE) (Wang et al., 2019b) is as low as $10^{-3}$–$10^{-4}$. Therefore, the practical application of haploid-inducer methods is likely very limited without a reporter or a visible phenotype, a direction that should be pursued as any recipient lines can generate the targeted mutation, thus avoiding an additional transformation step (Kelliher et al., 2019). Our SFR system also provides a promising solution for species that are difficult to transform. Indeed, in all cases the fluorescent marker allows for an easy selection of seeds carrying the CRISPR/Cas9 cassette even with very low transformation efficiencies. After the identification of positive transformants, the selection of progeny that have lost the transgene, as evidenced by the loss of red fluorescence, would allow for a facile method to obtain genome-edited and transgene-free plants from any germplasm.

Differences between En- and Em-SFR/Cas9 systems

The En-SFR seed sorting can be directly performed on maize ears, whereas Em-SFR seeds would in theory require prior threshing to expose the embryos, which would otherwise be hidden by neighboring kernels. Since all ears eventually need to be threshed, this difference may be ignored in practice. Another important point to consider is the genomic origin of gametes harboring SFRs. For En-SFR, most embryos share the same genome composition as the endosperm, as the central nucleus is derived from the same haploid tetrad cell as the egg cell, while the two sperm nuclei involved in double fertilization are genetically identical. However, hetero-fertilization (Sprague, 1929), whereby the egg cell and the central cell from the same ovule are fertilized separately by two independent sperm cells, may occur with a mean frequency of 1.46% in maize (Gao et al., 2011). Therefore, we speculate that, on rare occasions, an En-SFR-positive phenotype may not be consistent with the embryo genotype. We did not observe a single instance of this phenomenon in the current study. Hetero-fertilization may thus not be a major concern for the implementation of the Em-SFR system.
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Is genome editing using a DNA-free method or transient expression systems compatible with the SFR strategy

The SFR strategy is useful to isolate genome-edited, transgene-free plants. However, gene editing through “DNA-free” or transient expression systems is currently out of reach of the SFR approach. For example, gene editing has been achieved in plants with Cas9-gRNA ribonucleoprotein (RNP) complexes (Woo et al., 2015; Svitashhev et al., 2016; Toda et al., 2019). However, RNP is technically challenging. Moreover, RNP methods need to identify edited plants from a large population mixed in with unedited plants since there are no antibiotics/herbicides to provide selection pressure. To date, genome editing through a stable transformation strategy is the main application for SFR approaches.

MATERIALS AND METHODS

SFR/Cas9 vector construction

The SFR expression cassette was combined with the CRISPR/Cas9 stable expression cassette (Figure 1). An embryo-specific promoter of Zm.3896 (Liu et al., 2014) designated ZmESP, was PCR-amplified from genomic DNA of maize inbred line B73. The barley (Hordeum vulgare) endosperm aleurone cell-specific promoter HvASP (from Lipid Transfer Protein 2 (Ltp2)) (Kalia et al., 1994) was PCR-amplified from genomic DNA extracted from the spring barley cultivar Morex. DsRED2 was used as the red fluorescence protein for both En-SFR and Em-SFR systems. Sequence details about the key elements of promoters and the DsRED2 gene are provided in our previous reports (Dong et al., 2018; Qi et al., 2020b).

The CRISPR/Cas9 expression cassette was modified from our previously reported version (Li et al., 2017). Briefly, the modified SpCas9 sequence was cloned into the carboxypeptidase B vector. In addition, the DNA segments from nucleosimplin and Simian Virus 40 (SV40) encoding their nuclear localization signals (NLS) were cloned 5′ or 3′ of SpCas9, respectively. The efficiency of CRISPR/Cas9 has been previously validated (Li et al., 2017, 2019; Dong et al., 2018, 2019; Liu et al., 2020; Qi et al., 2020a). Expression of the sgRNA was driven by the maize U6-2 promoter, as previously described (Qi et al., 2018). A total of 14 transcription factor genes were selected as targets to generate knockout mutations within the coding region by using SFR/Cas9 (Table 1). The B73 reference genome (B73_RefGen_v4) was used to design sgRNAs. The target sequences were confirmed to be intact in inbred line KNS585, used for transformation, via Sanger sequencing. One verified sgRNA design for each gene was introduced into the CRISPR/Cas9 vector for maize transformation.

Maize transformation

All constructs for plants transformation were introduced into Agrobacterium (Agrobacterium tumefaciens) EHA105 strain. Immature maize embryos from inbred KNS585 were transformed with the CRISPR/Cas9 constructs by Agrobacterium-mediated stable transformation. Transformations and plant regeneration were performed through a commercial service provided by Weimi Biotechnology Co., Ltd (Changzhou, Jiangsu, China).

One En-SFR/Cas9 (En0 plasmid) and one Em-SFR/Cas9 (Em0 plasmid) were used for independent transformations (Tables S1, S2). In addition, one 12-plasmid pool consisting of six En-SFR/Cas9 plasmids (En1-6) and six Em-SFR/Cas9 plasmids (Em1-6) was used for another transformation. Agrobacterium colonies harboring each plasmid were allowed to grow at 28°C in Luria–Bertani medium until they reached an optical density (OD600) of approximately 0.3. All Agrobacterium cultures were then pooled in equal volumes.

Imaging and fluorescence observation of SFRs

Maize seed kernels, ears and whole plants were photographed using a Canon 70D digital camera (Canon, Japan). The ears and kernels of En-SFR/Cas9, Em-SFR/Cas9, and Em/En-SFR/Cas9 transformants were observed and photographed under a FL5000 inspection light (NIGHTSEA LLC, Lexington, MA, USA) equipped with a 550 nm excitation wavelength/filter. Longitudinal sections of SFR/Cas9-positive seeds were photographed after excitation at 550 nm with a Nikon stereomicroscope (SMZ1500; Japan) at 7.5x magnification. Observations of positive seeds at single cell resolution were performed with a Zeiss microscope ( LSM700; Germany) with a 554 nm excitation wavelength at 100x magnification.

Validation of maize transformation events

Plant genomic DNA was extracted from putative transformants using a Plant Genomic DNA Kit (Tiangen, China) according to the manufacturer’s protocol. The presence/absence of the T-DNA cassette was determined with the Biaplaphos (Bar) strip test (Catalog No. STX14200/0012; Agdia, USA) and by PCR amplification of the SpCas9 gene with primers 5′-CAACCGGAAAGTGACCGTG-3′ and 3′-CACCCACACTGCTCGA-3′. The PCR program consisted of an initial denaturation at 94°C of 3 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 68°C for 20 s, and a final extension at 68°C for 10 min.

Sanger sequencing of target mutations

Sanger sequencing was performed to identify the mutations introduced by genome editing in the target genes on an ABI 3730 (Applied Biosystems, Foster City, CA, USA). Primers for PCR and sequencing are listed in Table S2. The sequencing chromatograms were viewed with Snapgene Software v. 2.0.1. The functional consequences of the detected mutations were analyzed by using the web-based tool DSDecode (Liu et al., 2015) or via artificial DNA sequence alignment.

Single-target and small-scale mutant libraries

For putative En0 and Em0 transformants, kernels that were positive for red fluorescence were selected among T2 seeds and planted in soil to set T1 seeds. The T1 progeny was then subjected to another round of selection, this time looking for kernels with no detectable red fluorescence indicative of the absence of
the original transgene, followed by genotyping to confirm the presence of the desired target mutation.

Likewise, for putative transformants from the pooled 12 SFR/Cas9 plasmids, T₀ kernels were divided into En-, Em-, and Em/En-SFR subpools according to the red fluorescence pattern of the seed endosperm and/or embryo. All selected fluorescent T₀ seeds were planted to collect T₁ seeds. Individual T₁ plants from each subpool were analyzed for the presence of the SFR/Cas9 vectors by nested PCR amplification from genomic DNA, with two primer pairs annealing to sequences covering the ZmU6 promoter and the sgRNA scaffold region. The primer sequences were first forward primer: 5’-GGTTCATGCGTTCACAGGTGC-3’, first reverse primer: 5’-TGGTAGAGGGTGCTGAGG-3’; and second forward primer: 5’-GGATGTCGCGTCTCCCTGAATA-3’, second reverse primer: 5’-TCAGTTGTAACGGACTAGCGCT-3’. The first PCR products were diluted 2.5-fold and used as templates for the second round of nested PCR. The first PCR program consisted of 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 58 °C for 30 s, 68 °C for 1 min 30 s, and a final extension at 68 °C for 10 min. The second PCR program had an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 68 °C for 20 s, and a final extension at 68 °C for 10 min. The second nested PCR products were separated on agarose gel, excised, purified, and cloned with the pEASY®-Blunt Cloning Kit (#BM111-02; TransGen Biotech. Co. Ltd, Beijing, China). The sequence of the insert from individual clones was determined with M13F primer on an ABI 3730 sequencer (Applied Biosystems). Each T₁ plant was then subjected to single or multiplex mutation identification by Sanger sequencing. T₁ seeds harboring the target mutations but without the SFR constructs (as evidenced by the loss of red fluorescence) were selected for further phenotyping or breeding applications.

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CONFLICT OF INTEREST

The authors declare there are no conflict of interests.

AUTHOR CONTRIBUTIONS

C.X. designed the research; Y.Y., J.Z., X.Q., and C.L. conducted the experiments; C.L., B.C., and C.X. supervised the research; C.X. and C.L. wrote the manuscript.
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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.13086/supportinfo

Figure S1. Cross-verification of SFR/Cas9 +/- sorted seeds with the Bar stripe test and SpCas9 polymerase chain reaction (PCR) detection M, molecular marker (#BM111-02, Trans2K® Plus DNA Marker; TransGen Biotech Co. Ltd, Beijing, China). Bialaphos (Bar) strip test (Catalog No. STX14200/0012; Agdia, USA). Scale bar = 1 cm.

Table S1. List of constructed vectors using Em and/or En-SFR-Cas9 in this study

Table S2. Constructed plasmids for generating target gene mutations and mutation identification polymerase chain reaction (PCR) details

Table S3. High efficient identification of “transgene-free” Zm1g1 mutant from a single Em-SFR/Cas9 transformation

Table S4. High efficient identification of “transgene-free” Zm00001d024-337 mutant from a single Em-SFR/Cas9 transformation

Table S5. Full list of generated single- or multiplex mutants through subpooling strategy using Em- and/or En-SFR/Cas9

Table S6. Generation of single- or multiplex mutants through subpooling strategy using Em- and/or En-SFR/Cas9
Seed sorting accelerated genome editing