

# Comparative Study Between the CRISPR/Cpf1 and CRISPR/Cas9 systems for Multiplex Gene Editing in Maize

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## Methodology article

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# Abstract

**Background:** The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system has been successfully used for multiplex gene editing in crops. Although CRISPR/Cas9 system has been proved to be an efficient multiplex gene editing system in crops, it was still unclear how CRISPR/Cpf1, a natural direct repeat (DR)-based multiplex gene editing system, performed in crops. To this end, this study compared the CRISPR/Cpf1 system and CRISPR/Cas9 system for multiplex gene editing in maize.

**Results:** The bZIP transcription factor *Opaque2* (*O2*) was used as the target gene to evaluate the editing efficiency of both systems. We found that in the T0 generation, the CRISPR/Cpf1 system showed low editing efficiency with only one mutation, while the CRISPR/Cas9 system generated many different types of on-target mutations. In the T1 generation, the CRISPR/Cpf1 system still showed lower editing efficiency than the CRISPR/Cas9 system. However, in the T2 generation, the CRISPR/Cpf1 system generated more types of new mutations. While the CRISPR/Cas9 system tended to edit within the on-target range, the CRISPR/Cpf1 system preferred to edit in between the targets. We also found that in the CRISPR/Cpf1 system, the editing efficiency positively correlated with the expression level of Cpf1.

**Conclusions:** In conclusion, the CRISPR/Cpf1 system offers alternative choices for target-site selection for multiplex gene editing and has acceptable editing efficiency in maize. Thus, the CRISPR/Cpf1 system is a valuable alternative choice for gene editing in crops.

## Background

Gene editing technologies can efficiently create mutations in targeted genes, which are important in basic and applied biological research. The CRISPR/Cas system, among many gene editing technologies, is one of the most popular systems due to its simple process of vector construction and high editing efficiency [1–6]. CRISPR/Cas9 is a functionally prioritized editing system from *Streptococcus pyogenes* (Sp) and has been successively applied to endogenous genome editing in multiple organisms [6–12]; however, it is a natural one-unit system and cannot target multiple targets without modifications [2, 3].

Multiplex gene editing with the CRISPR/Cas system is of great value, as it is expected to greatly facilitate crop genome engineering and precision breeding [2, 13, 14]. Multiplexing is mainly achieved by introducing multiple gRNAs *in vivo* using multigene cassette gRNA expression [15], Csy4-based excision [16], ribozyme-flanked gRNAs [17], or tRNA-based cleavage of gRNAs [18]. Among those, the tRNA-based system was thoroughly tested from a single transcript with high editing efficiency in crops [18–20]. However, its overlong tRNA-based units, complexity of vector construction and low efficiency of plant transformation have limited its ability in multiplex gene editing.

In contrast to SpCas9, Cpf1 is a class 2 natural multiunit CRISPR/Cas system that was recently discovered [21] and has been consecutively utilized for gene editing in plants [22–25]. Compared to the CRISPR/Cas9 system, the CRISPR/Cpf1 system has many distinct features. Firstly, CRISPR/Cpf1 is a

natural multi-unit system with a simple short DR-based unit itself. As Cpf1 nuclease has a crRNA processing activity, it simplified the constructive process of multiplex gene editing [26, 27]. Secondly, since the CRISPR/Cpf1 system has a short crRNA and a smaller Cas protein, it could handle larger vector loads, and is thus more suitable for multiple targets editing [21, 26, 28]. Thirdly, CRISPR/Cpf1 is a single RNA-guided endonuclease system that only needs one crRNA and does not require an additional tracrRNA which is critical in the CRISPR/Cas9 system [21, 29, 30]. Furthermore, Cpf1-crRNA complexes could efficiently cleave target DNA preceded by a short T-rich TTTN (N = A, C or G) PAM, while the CRISPR/Cas9 system targeted the G-rich PAM following the target DNA [21]. Moreover, Cpf1 cleaves DNA and introduces a staggered DNA double-stranded break with a 4- or 5-nt 5' overhang, which increases the probability of homology-directed repair pathways and facilitates site-specific insertion and replacement of DNA fragments [21, 27]. Although it has been shown that the CRISPR/Cpf1 system can edit genes using a single crRNA in maize [31], there has not yet been a study on its multiplex gene editing capability in crops.

Maize (*Zea mays* L.) is one of the most important cereal crops in the world. Using it as a model, we evaluated the multiplex gene editing efficiency of CRISPR/Cpf1 compared to the CRISPR/Cas9 system. We designed a CRISPR/Cpf1 system with our specific vector design, which was optimized for its usage in maize. We found many advantages of this design and thus potentially provided an innovative strategy for multiplex gene editing in crops.

## Results

# Multiplex gene editing vector design based on the CRISPR/Cpf1 and CRISPR/Cas9 systems in maize

To evaluate the performance of the multiplex gene editing CRISPR/Cpf1 system in maize, we compared its efficiency against that of the CRISPR/Cas9 system using the same codon-optimization strategy for their better expression in maize. We cloned a maize RNA polymerase (Pol) III promoter as pZmU6 to drive crRNAs for the CRISPR/Cpf1 system or sgRNAs for the CRISPR/Cas9 system also for their better expression in maize, together with tZmU6 (Fig. 1a, Additional file 1: Fig. S1). In these expression cassettes, we designed and synthesized the sequence of the crRNA expression cassette for the CRISPR/Cpf1 system and the sgRNA expression cassette for the CRISPR/Cas9 system (Fig. 1b, Additional file 1: Fig. S1). Multiple crRNAs were separated by a simple short DR-based unit for the CRISPR/Cpf1 system, and multiple sgRNAs were separated by a tRNA-based unit for the CRISPR/Cas9 system (Fig. 1a).

The maize *O2* gene is a gene involved in mutant kernels with the opaque phenotype. We used it as the target gene to examine the feasibility of the CRISPR/Cpf1 and CRISPR/Cas9 systems. We used CRISPR-GE (<http://skl.scau.edu.cn/>) to select target sites [32]. For a fair comparison, we selected target sites in the same region of the *O2* gene locus as close as possible. The regions including three targets of the CRISPR/Cpf1 and CRISPR/Cas9 systems substantially overlapped (Fig. 1c). After altering series of

effector factors, including guanine-cytosine content, region and potential off-target sites, as well as pairing with crRNA (sgRNA in the CRISPR/Cas9 system), we targeted three sites in the 2nd exon of the *O2* gene with both the CRISPR/Cpf1 and CRISPR/Cas9 systems.

## Acquisition of transgenic plants for the CRISPR/Cpf1 and CRISPR/Cas9 systems

For the CRISPR/Cpf1 and CRISPR/Cas9 systems, we obtained five transgenic lines by the *Agrobacterium*-mediated maize transformation method. We chose three lines that had a sufficient number of progenies. At the T0 generation, two regenerated transgenic plants per line were examined. We utilized specific primers for the *bialaphos resistance (Bar)* gene to verify all transgenic plants of the two systems (Additional file 1: Fig. S3, Table. S1).

The transcript expression levels of Cpf1 and Cas9 nucleases were analyzed by qRT-PCR (Fig. 2a, 2b, Additional file 1: Table. S1). We found that the expression levels of *Cas9* in line 1 and line 3 were similar, while they were approximately sixty times higher in line 2 (Fig. 2a, Additional file 1: Fig. S4b). For *Cpf1*, the expression level in line 1 was the highest, followed by line 2 and line 3 (Fig. 2b, Additional file 1: Fig. S4c). Transcript expression verified the transgenic plants, and the expression levels contained differences between lines.

### CRISPR/Cpf1 systems showed lower target gene editing efficiency than CRISPR/Cas9 in the T0 and T1 generations

To compare the editing efficiency between the CRISPR/Cpf1 and CRISPR/Cas9 systems, genomic DNA was isolated from leaf tissues of T0 plants. A 1481 bp region of *O2* including all targets was amplified by PCR using primers ZmO2-exon2-F and R for sequencing and assembly (Additional file 1: Table. S1). The PCR products were either directly subjected to Sanger sequencing or cloned into pMD18-T and then subjected to colony sequencing. Sequencing results showed that all three lines of the CRISPR/Cas9 system contained editing results. Among the three lines, the 1st target was vulnerable to editing; for instance, it contained a 1 bp (T) deletion or insertion in different plants. The 2nd target contained a 1 bp (T) insertion and 21 bp deletions displayed on the 3rd target (Fig. 3a, Table 1). However, in the CRISPR/Cpf1 system, only the two seedlings in line 1 contained a 12 bp insertion between the 1st and 2nd targets (Fig. 3b, Table 2). Therefore, in maize, the CRISPR/Cas9 system is more effective than CRISPR/Cpf1 for multiplex gene editing in the T0 generation.

We then chose each of three lines from the CRISPR/Cpf1 and CRISPR/Cas9 systems for the T1 generation. The T1 progenies were produced by crossing the pollen of T0 lines to the W22 inbred line. At the T1 seedling stage, gene editing of the *O2* gene was analyzed by PCR and sequencing analysis. Consistent with our expectation, all three lines from the CRISPR/Cas9 system contained editing results. The results showed that nineteen of thirty seedlings of line 3 contained editing results at the highest editing efficiency. The secondly high editing efficiency was in line 1; fifteen of thirty seedlings contained editing results. Eleven of all thirty seedlings of line 2 contained editing results (Additional file 1: Fig. S4b).

Among the three lines, we verified that a number of mutations were inherited from the last generation, for instance, a 1 bp (T) deletion and insertion (Fig. 3a, Table 1). Furthermore, one plant newly contained a 1 bp (T) deletion on the 1st target and a 1 bp (T) insertion on the 2nd target as well as a 1 bp (G) insertion on the 3rd target (Fig. 3a, Table 1).

In terms of the CRISPR/Cpf1 system, four out of all thirty seedlings of line 1 contained editing results. As mentioned in the T0 generation, the same editing result, a 12 bp fragmental insertion, was observed, indicating that it was inherited (Fig. 3b, Table 2). Three out of all thirty seedlings of line 2 contained editing results, which indicated that there were no mutations in the T0 generation, and new mutations, such as a 3 bp deletion and a 2 bp insertion between the 2nd and 3rd targets, were generated in the T1 generation (Fig. 3b, Table 2). Line 3 continued to not contain editing result in the T1 generation (Additional file 1: Fig. S4c). Thus, the CRISPR/Cpf1 system still shows lower editing efficiency than the CRISPR/Cas9 system in the T1 generation. The results illustrated that the editing positions of the CRISPR/Cpf1 system mostly occurred between the two targets instead of within the common on-target region. Furthermore, the Cas9 nuclease has no relationship between editing efficiency and its transcript levels, while the editing efficiencies of each line in the CRISPR/Cpf1 system were directly and proportionally correlated with the level of Cpf1 nuclease transcript expression (Additional file 1: Fig. S4b, Fig. S4c).

Table 1  
The DNA sequence of mutations displayed in the CRISPR/Cas9 system.

|    | Target sequence 1 (5'-3')        | In/Del  | Symbol             |
|----|----------------------------------|---------|--------------------|
| WT | GGAGATCCTCGGGCCCTTCT <b>GGG</b>  | /       | /                  |
| T0 | GGAGATCCTCGGGCC---CT <b>GGG</b>  | -3 bp   | D <sup>3a*</sup>   |
|    | GGAGATCCTCGGGCCCTTTCT <b>GGG</b> | + 1 bp  | I <sup>1a*</sup>   |
|    | GGAGATCCTCGGGCCCT-CT <b>GGG</b>  | -1 bp   | D <sup>1a*</sup>   |
| T1 | GGAGATCCTCGGGC---TCT <b>GGG</b>  | -3 bp   | D <sup>3b</sup>    |
|    | GGAGATCCTCGGGCCAT-CT <b>GGG</b>  | R;-1 bp | R; D <sup>1b</sup> |
|    | GGAGATCCTCGGGCC--TCT <b>GGG</b>  | -2 bp   | D <sup>2</sup>     |
|    | GGAGATCCTCGGG----- <b>GGG</b>    | -7 bp   | D <sup>7</sup>     |
|    | GGAGATCCTCGGGCC-----             | -23 bp  | D <sup>23</sup>    |
|    | TATT-----CCAC                    | -60 bp  | D <sup>60</sup>    |
|    | GTGG-----GGTG                    | -200 bp | D <sup>200</sup>   |
|    | Target sequence 2 (5'-3')        | In/Del  | Symbol             |
| WT | GTGGACCTTTGAGAGGTTACT <b>GG</b>  | /       | /                  |
| T0 | GTGGACCTTTGAGAGGTTTACT <b>GG</b> | + 1 bp  | I <sup>1b*</sup>   |
|    | GTGGACCTT-----ACT <b>GG</b>      | -9 bp   | D <sup>9*</sup>    |
| T1 | GTGG-----GGTG                    | -200 bp | D <sup>200</sup>   |
|    | Target sequence 3 (5'-3')        | In/Del  | Symbol             |
| WT | GGTAATGATGGCGCCTGCGG <b>CGG</b>  | /       | /                  |
| T0 | -----GCG <b>G</b>                | -21 bp  | D <sup>21</sup>    |
| T1 | GGTAATGATGGCGCCTG <b>GCGGCGG</b> | + 1 bp  | I <sup>1c</sup>    |
|    | GGTAATGATGGCGCCTG <b>ACGGCGG</b> | + 1 bp  | I <sup>1d</sup>    |
|    | GGTAATGATGGCGCCT- <b>CGGCGG</b>  | -1 bp   | D <sup>1c</sup>    |

I/D, insertions and deletions. R, replacement. Different I/D results are numbered 1 (then 2, 3 ...). The PAM sequence, base insertions and base replacements are bolded, base deletions are marked with dashes. These symbols linked with Fig. 3a.

Table 2  
The DNA sequence of mutations displayed in the CRISPR/Cpf1 system.

|     | Sequence (5'-3')                        | In/Del   | Symbol                           |
|-----|---|----------|----------------------------------|
| WT  | AGAGCCAGAGCGAGAGC                       | + 12 bp  | I <sup>12*</sup>                 |
| M1  | AGAGCCAGAG <b>CCAGAGCCAGAG</b> CGAGAGC  |          |                                  |
| WT  | GTATATA <b>CACTGCTCGCTCTT</b>           | R1;+2 bp | R <sup>a</sup> ; I <sup>2*</sup> |
| M2  | GTATATA <b>TACAATGCTCGCTCTT</b>         |          |                                  |
| WT  | CGGTGGTGGTGGT <b>GCCGAA</b>             | -3 bp    | D <sup>3</sup>                   |
| M3  | CGGTGGTGGTG <b>---</b> CCGAA            |          |                                  |
| WT  | TCCCTTTCTTGAC <b>TTTGCTT</b>            | -1 bp    | D <sup>1a</sup>                  |
| M4  | TCCCTT <b>—</b> CTTGAC <b>TTTGCTT</b>   |          |                                  |
| WT  | AC <b>TTTGCTT</b> GGAACCATTGA           | R2;-1 bp | R <sup>b</sup> ; D <sup>1b</sup> |
| M5  | AC <b>TTTG</b> CA <b>TG—</b> AACCATTGA  |          |                                  |
| WT  | TCTGGGAGCTGCTACCACCG                    | -1 bp    | D <sup>1c</sup>                  |
| M6  | TCTGGGAGCTGCTAC <b>—</b> ACCG           |          |                                  |
| WT  | CCGAC.....AGAGCGAG                      | -200 bp  | D <sup>200</sup>                 |
| M7  | CCGAC <b>—</b> ..... <b>—</b> AGAGCGAG  |          |                                  |
| WT  | GCTGCTGGTCATGGT <b>GACGG</b>            | -1 bp    | D <sup>1d</sup>                  |
| M8  | GCTGCT <b>—</b> GTCATGGT <b>GACGG</b>   |          |                                  |
| WT  | <b>TTTGAGAGGTTACTGGAAGAGGAG</b>         | R3       | R <sup>c</sup>                   |
| M9  | <b>TTTGAGAGGTTACTG</b> <b>AAAGAGGAG</b> |          |                                  |
| WT  | CGGTGGTGGTGGT <b>GCCGAAC</b>            | -1 bp    | D <sup>1e</sup>                  |
| M10 | CGGTGGTGGTG <b>—</b> TGCCGAAC           |          |                                  |

I/D, insertions and deletions. R, replacement. Different I/D results are numbered 1 (then 2, 3 ...). The PAM sequence, base insertions and base replacements are bolded, base deletions are marked with dashes. These symbols linked with Fig. 3b.

To summarize, we managed to perform multiplex gene editing with the CRISPR/Cpf1 system with multiple crRNAs and compared with that of the CRISPR/Cas9 system. Our results showed that the editing efficiency based on multiple gRNAs of the CRISPR/Cas9 system was superior to that of multiple crRNAs of the CRISPR/Cpf1 system.

## **CRISPR/Cpf1 generated more types of new mutations in the T2 generation**

According to the sequencing results in the T0 and T1 generations, the CRISPR/Cpf1 system showed relatively poor editing efficiency and uncommon editing patterns. Compared to other CRISPR systems, whose editing sites were usually on-target regions, its editing sites located between the two targets. Moreover, it precisely protected the target sites from cleavage and could implement continuous editing in the next generation.

To further explore the value of the CRISPR/Cpf1 system in maize, we further examined four ears of each line edited by the CRISPR/Cpf1 system in the T2 generation. Half of ears of line 1 and 2 contained editing results, while other ears were not. No editing events were found in line 3 ears. To evaluate the editing efficiency of the CRISPR/Cpf1 system, we selected eight kernels of each ear for plantation. We found that the CRISPR/Cpf1 system created more types of new mutations (Fig. 3b, Additional file 1: Fig. S4a, Fig. S4c). For instance, a 1 bp deletion was displayed between the 1st and 2nd targets and between the 2nd and 3rd targets as well as on the 1st target (Fig. 3b, Table 2). It also created a base replacement on the 1st target and 2nd target (Fig. 3b, Table 2). Surprisingly, there was a 200 bp longer fragmental deletion including the 1st target (Fig. 3b, Table 2). In short, for the CRISPR/Cpf1 system, we observed more types of new mutations in the next generation.

## **Generation of opaque phenotypic mutants**

Vitreous and opaque kernels in the same ear were selected for further phenotyping from the CRISPR/Cpf1 and CRISPR/Cas9 systems (Fig. 4). Generally, maize mature starchy endosperm is divided into an external vitreous region and a starchy internal region. If vitreous regional development is affected, it may result in an opaque or floury phenotype [33, 34]. Under normal light, mutant kernels (*O2-Mu*) showed a distinct opaque phenotype, and wild-type kernels (*O2-WT*) displayed a vitreous endosperm phenotype in the two systems (Fig. 4a, Fig. 4b). An opaque phenotype indicated that our Cpf1 nuclease was correctly transferred and functioning in maize.

## **Discussion**

In the last few years, the CRISPR technologies had greatly speeded up both research and breeding of maize. As it has shown that both the CRISPR/Cas9 and CRISPR/Cpf1 systems were efficient for multiplex gene editing in rice [35], CRISPR/Cpf1 system, the natural direct repeat (DR)-based multiplex gene editing system, might be a potentially useful system in maize. In this study, we developed a strategy to design, synthesize and utilize multiple natural units for multiplex gene editing with CRISPR/Cpf1 in maize (Fig. 1)

and compared it to the CRISPR/Cas9 system (Fig. 1) [20]. A previous study indicated that in maize, the editing efficiency based on a single crRNA of the CRISPR/Cpf1 system was less effective than that of the CRISPR/Cas9 system [31]. It was reported that, different construction strategies, including promoter selection, target selection and Cas nuclease codon optimization, might influence the editing efficiency [36]. The RNA Pol III promoters showed high specialization for the production of short noncoding RNAs and were commonly used to express crRNAs or sgRNAs in CRISPR/Cas systems in previous studies [37]. We utilized pZmU6 as an RNA Pol III promoter to express crRNAs or sgRNAs for their better expression in maize (Fig. 1). We used maize codon-optimized Cas nucleases for their better expression in maize. We found that, consistent with the previous study, the editing efficiency of multiple crRNAs for the CRISPR/Cpf1 system using DR-based units was less effective than that based on multiple gRNAs for the CRISPR/Cas9 system in maize (Additional file 1: Fig. S4). However, after using the RNA Pol III promoter and the maize codon-optimized Cas nucleases, we found that the CRISPR/Cpf1 system could generate an increased number of editing events in the T2 generation (Additional file 1: Fig. S4). This indicated that the CRISPR/Cpf1 system with a suitable construction strategy still obtained acceptable editing efficiency in maize.

We found that the editing position of the CRISPR/Cpf1 system mostly occurred between the two targets instead of within the common on-target region (Fig. 3). The Cas9 nuclease acted mostly within the on-target region, and once the target was destroyed, it was difficult to continue editing in the next generation [8, 15, 38, 39]. However, in the CRISPR/Cpf1 multiplex gene editing system, the targets were protected from cleavage to the extent that it could introduce continuous editing in the next generation.

Additionally, we found that the editing efficiency of CRISPR/Cpf1 system positively correlated with the level of Cpf1 nuclease transcript expression, which did not exist in the CRISPR/Cas9 system (Additional file 1: Fig. S4). Based on this observation, we could use the Cpf1 expression level as an indicator to select lines for downstream analysis before obtaining the editing results. This could improve the efficiency of editing verification.

## Conclusions

This is the first report of successful multiplex gene editing using the CRISPR/Cpf1 system in maize. In this study, we utilized pZmU6, DR-based units and maize codon-optimized Cpf1 to design and introduce multiplex gene editing with the CRISPR/Cpf1 system in maize. After optimized the system for maize, we found many different types of mutations were produced in the T2 generation, despite of the low efficiency in the T0 and T1 generation compared to the CRISPR/Cas9 system. We found features of the CRISPR/Cpf1 system in which the editing site was mostly between targets, and a variety of new mutations were produced in the next generation. In addition, the Cpf1 transcript expression level was positively correlated with its editing efficiency. In summary, the CRISPR/Cpf1 system can be used in maize with acceptable editing efficiency and offers an alternative choice for target-site selection.

## Methods

# Plant materials

The maize (*Zea mays L.*) parental line Hi II was used in this study. Hi II was initially acquired from the Maize Genetics Cooperation Stock Center and cultured in the laboratory. All the transgenic lines were obtained in the laboratory. Maize plants were cultivated in the experimental field and greenhouse at the campus of Shanghai University.

## Construction of CRISPR/Cpf1 and CRISPR/Cas9 vectors

The CRISPR/Cpf1 and CRISPR/Cas9 vectors of this study are shown in a diagram in Fig. 1a. The Pol III promoter of pZmU6 and its tZmU6 were amplified by using gene-specific primers (Additional file 1: Table. S1) and cloned into the *Hind*III site of the pCAMBIA3301 vector. The maize codon-optimized *Cpf1* gene and maize codon-optimized *Cas9* gene were cloned into the abovementioned vector (Fig. 1a). The *Bar* gene was driven by the 35S promoter for the selection of transgenic plants on pCAMBIA3301 (Fig. 1a). We optimized the maize codon of *Cpf1* (Additional file 1: Fig. S2), and the sequence of maize codon-optimized *Cas9* was shown in our previous study [20]. The pZmUbi and Nos terminator (Nos T) were utilized to express Cpf1 or Cas9. The multi-crRNA expression cassette designed for multiplex editing was synthesized by Generey (Generey.com) and cloned into the *Bss*HII sites between pZmU6 and tZmU6 of the vector (Fig. 1a, Fig. 1b, Additional file 1: Fig. S1). A nuclear localization signal (NLS) was added to both ends of Cpf1 or Cas9 (Fig. 1a). The final vector plasmid, pCAMBIA3301, was constructed and used for *Agrobacterium*-mediated maize transformation [40]. For specific construction of the CRISPR/Cas9 vector, please refer to our previous study [20].

### **Agrobacterium -mediated immature maize embryo transformation**

*Agrobacterium*-mediated maize transformation was carried out according to Frame et al. [40]. All six independent transgenic lines of the CRISPR/Cpf1 and CRISPR/Cas9 systems were generated for each transformation. We used PCR with specific primers to confirm *Bar* positivity for all transgenic plants (Additional file 1: Fig. S3, Table. S1).

## RNA extraction and RT-PCR analysis

Total RNA was extracted from seedlings of both WT and mutant maize T1 transgenic plants using an RNA extraction kit (TIANGEN). Reverse transcription of the RNA was carried out by random primers. Specific primers used to amplify and quantify the Cpf1 and Cas9 genes are provided in the Supplemental material (Additional file 1: Table. S1). Total RNA (0.5 µg) from each sample was subjected to cDNA synthesis using the cDNA Synthesis Kit (Bio-Rad, Mississauga, Ontario, Canada) following the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed with the Light Cycler 480 real-time PCR system (Roche Diagnostics, Basel, Switzerland) using SsoFast EvaGreen Supermix (Bio-Rad, Mississauga, Ontario, Canada).

## Genomic DNA extraction and PCR/Sequencing assay

Maize genomic DNA was extracted from seedlings of T0 to T2 transgenic plants using the hexadecyltrimethylammonium bromide method [41]. PCR amplifications were performed with specific primer pairs flanking the designed target sites. Specific primers were used to amplify the *O2* gene to detect mutagenesis at the desired sites (Additional file 1: Table. S1). Taq Mix polymerase (Vazyme) was used in the amplification. Detailed methods can be found in a previous article [20].

## Abbreviations

CRISPR/Cas, clustered regularly interspersed short palindromic repeat/CRISPR-associated protein; DR, direct repeat; *O2*, *Opaque2*; *Sp*, *Streptococcus pyogenes*; gRNA, guide RNA; crRNA, CRISPR RNA; PAM, protospacer adjacent motif; pZmU6, maize U6 promoter; tZmU6, maize U6 terminator

## Declarations

### Ethics approval and consent to participate:

'Not applicable' in this section.

### Consent for publication:

'Not applicable' in this section.

### Availability of data and materials:

All data generated or analyzed during this study are included in this published article and its supplementary information files.

### Competing Interests:

The authors declare that they have no competing interests" in this section.

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### Authors Contributions:

RTS and WWQ conceived and designed research. CZG and SCH performed experiments. CZG collected and analyzed the data. RTS, WWQ and CZG wrote the manuscript. All authors read and approved the final manuscript.

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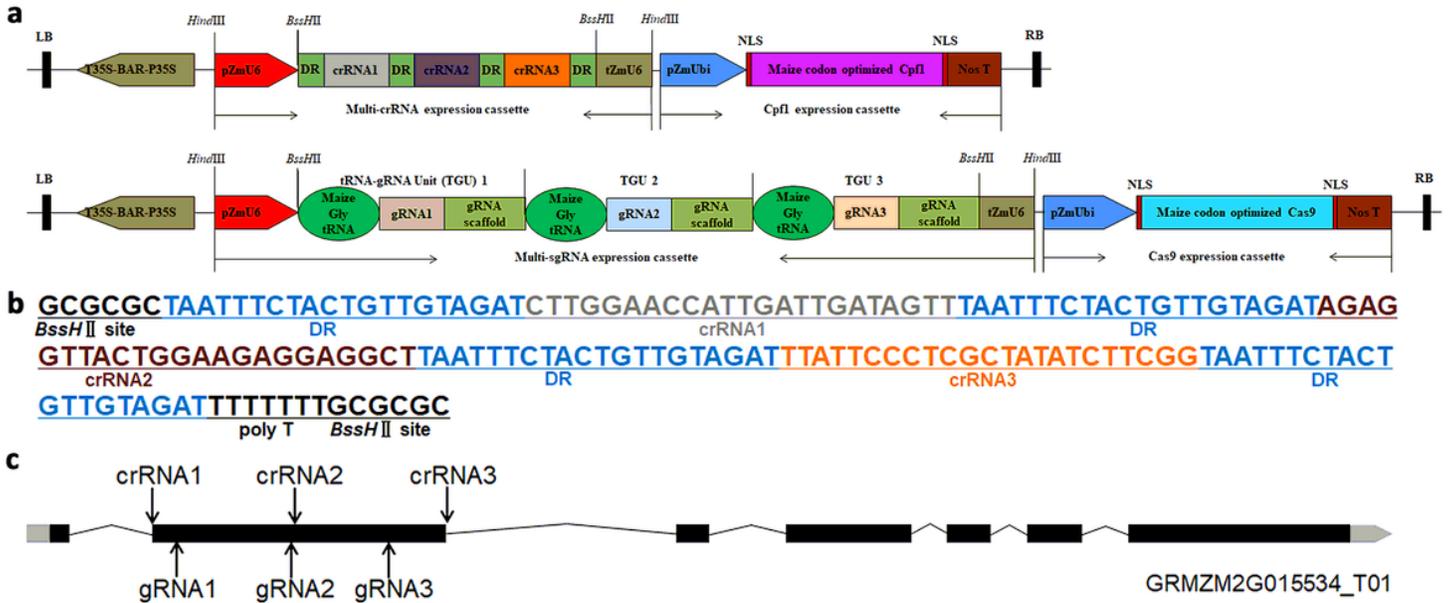
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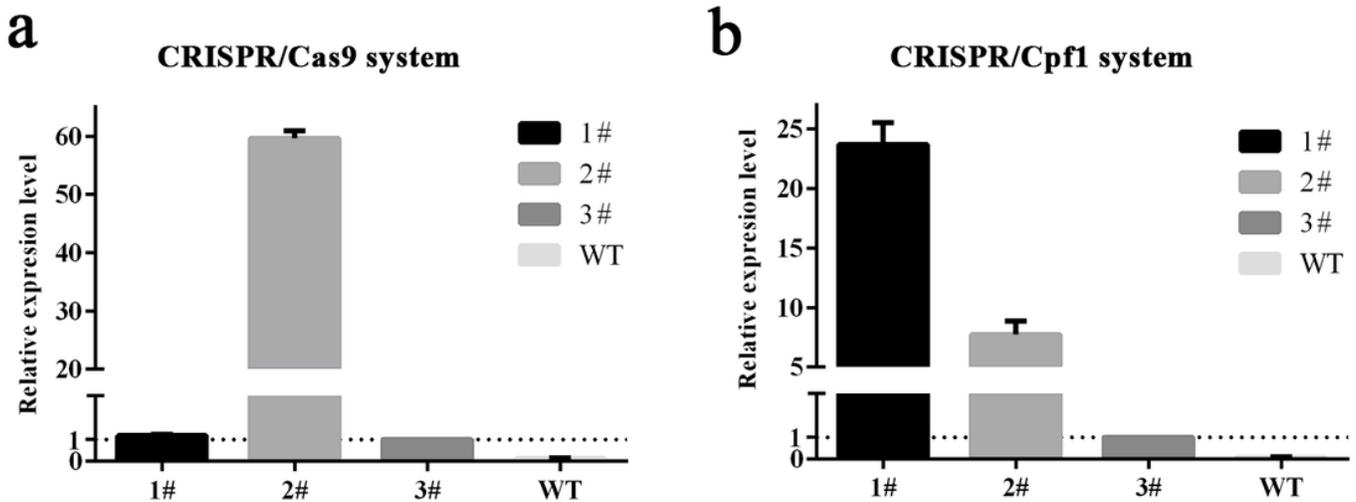
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## Figures



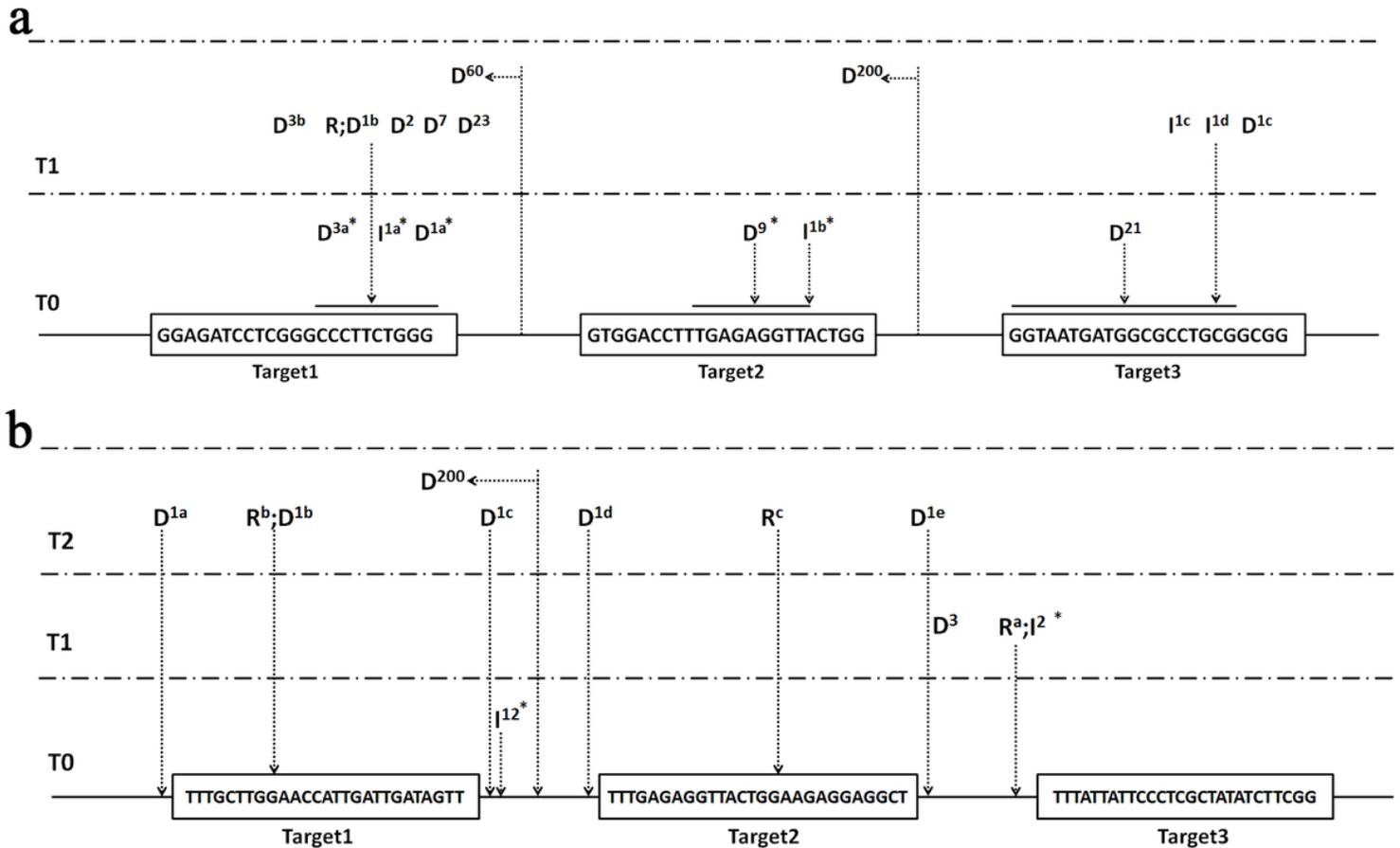
**Figure 1**

Strategy for vector construction of the multi-crRNA cassette and the target loci in two systems. (a) The strategy for vector construction of the CRISPR/Cpf1 and CRISPR/Cas9 systems on pCambia3301. pZmUbi, maize ubiquitin promoter; pZmU6, maize U6 promoter; tZmU6, maize U6 terminator; DR, direct repeat; NLS, nuclear localization sequence; Nos T, nopaline synthase terminator; BAR, phosphinothricin R; LB, left border; RB, right border; TGU, tRNA-gRNA unit. (b) The CRISPR/Cpf1 system was used to construct sequences of the multi-crRNA expression cassette for corporate synthesis. DR, direct repeat. (c) The loci of selected targets on the second exon of opaque2 (GRMZM2G015534\_T01) of the crRNAs or sgRNAs in the two systems.



**Figure 2**

Transcript expression levels of Cas nucleases in the CRISPR/Cpf1 and CRISPR/Cas9 systems. (a, b) The transcript levels of Cas9 and Cpf1 nucleases were determined by qPCR. WT, W22. Error bars are the SD for three biological replicates.



**Figure 3**

Mutation results of the CRISPR/Cpf1 and CRISPR/Cas9 systems in the generations. (a) The loci and lengths of the insertions and/or deletions (I/D) for the CRISPR/Cas9 system are presented. Different I/D results are numbered 1 (then 2, 3 ...). Inheritable mutations are marked with the asterisk (\*). R, replacement. The panel links with table 1. (b) The loci and lengths of the insertions and/or deletions (I/D) for the CRISPR/Cpf1 system are provided. Different I/D results are numbered 1 (then 2, 3 ...). Inheritable mutations are marked with the asterisk (\*). R, replacement. The panel links with table 2.

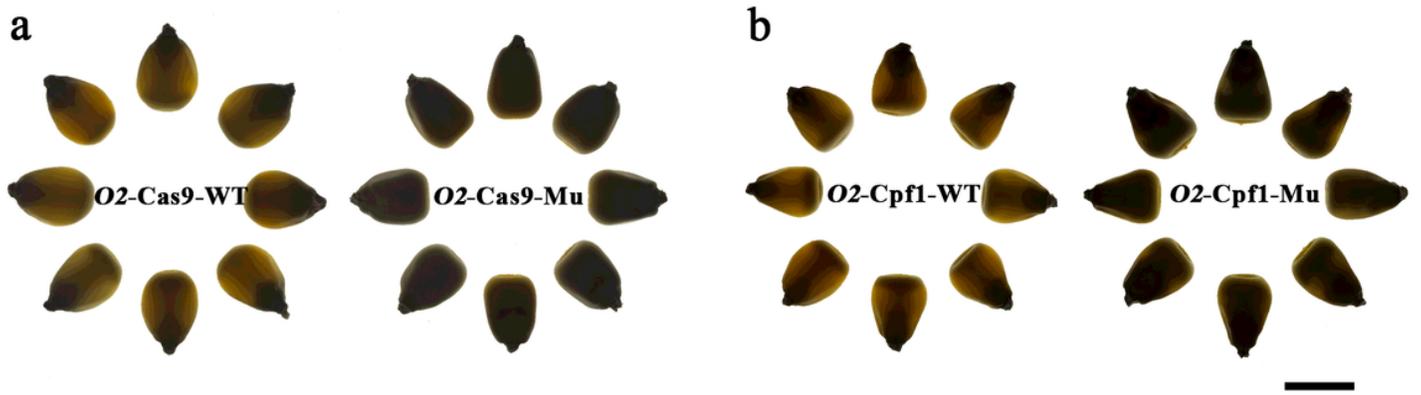


Figure 4

The opaque phenotype of mature kernels for the two systems in maize. (a, b) Mature (35 DAP) WT and T1 transgenic maize kernels are shown in the CRISPR/Cpf1 and CRISPR/Cas9 systems. Bar=1cm.

## Supplementary Files

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