Use of CRISPR/Cas9-Based Gene Editing to Simultaneously Mutate Multiple Homologous Genes Required for Pollen Development and Male Fertility in Maize

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Abstract: Male sterility represents an important trait for hybrid breeding and seed production in crops. Although the genes required for male fertility have been widely studied and characterized in many plant species, most of them are single genic male-sterility (GMS) genes. To investigate the role of multiple homologous genes in anther and pollen developments of maize, we established the CRISPR/Cas9-based gene editing method to simultaneously mutate the homologs in several putative GMS gene families. By using the integrated strategies of multi-gene editing vectors, maize genetic transformation, mutation-site analysis of T0 and F1 plants, and genotyping and phenotyping of F2 progenies, we further confirmed gene functions of every member in ZmTGA9-1/-2/-3 family, and identified the functions of ZmDFR1, ZmDFR2, ZmAcos5-1/-5, and ZmAcos5-2 in controlling maize male fertility. Single and double homozygous gene mutants of ZmTGA9-1/-2/-3 did not affect anther and pollen development, while triple homozygous gene mutant resulted in complete male sterility. Two single-gene mutants of ZmDFR1-2 displayed partial male sterility, but the double-gene mutant showed complete male sterility. Additionally, only the ZmAcos5-2 single gene was required for anther and pollen development, while ZmAcos5-1 had no effect on male fertility. Our results show that the CRISPR/Cas9 gene editing system is a highly efficient and convenient tool for identifying multiple homologous GMS genes. These findings enrich GMS genes and mutant resources for breeding of maize GMS lines and promote deep understanding of the gene family underlying pollen development and male fertility in maize.

Keywords: CRISPR/Cas9; gene editing; multiple homologous genes; pollen development; genic male sterility; maize

1. Introduction

Maize (Zea mays L.) is an important food and feed crop worldwide. As a fast growing C4 plant and a successful crop of heterosis utilization, it provides more than one-half of global calorie consumption [1]. In recent decades, the wide use of hybrid varieties has greatly increased maize yield. Manual or mechanical detasseling is required for maize hybrid seed production to prevent self-pollination. However, detasseling has obvious drawbacks, e.g., it is time-consuming, labor-intensive, expensive, affects plant growth, and reduces the yield of hybrid seeds [2]. Therefore, developing a male-sterility line is critical for producing hybrid maize seed.
Male sterility is a useful agronomy trait for hybrid seed production in crops, but its application in maize is not successful and has suffered from several problems. For example, cytoplasmic male sterility has been used in the production of commercial hybrid maize; however, due to the potential increase of disease susceptibility and unreliable restoration of cytoplasmic male sterility line, it is limited in producing hybrid seeds [3]. Environment sensitive genic male sterility has an unstable fertility risk under variable environments [4]. Genic male sterility (GMS) caused by nuclear genes alone can overcome the disadvantages mentioned above, but it suffers from producing large-scale GMS seeds through self-pollination. Fortunately, with technological advances, some biotechnology-based male-sterility systems with GMS genes have been developed successfully in crops, which includes seed production technology, multi-control sterility, and dominant male sterility in maize [5–7]. Thus, the isolation and identification of GMS genes has become a research focus, for developing biotechnology-based male-sterility systems in maize.

Thus far, more than 100 GMS genes have been identified and characterized in plants, most of which are reported to encode transcription factors (TFs) or lipid biosynthesis and transport proteins, including 49 in Arabidopsis, 38 in rice, and 24 in maize [2,8–10]. GMS genes isolated in maize, compared with Arabidopsis and rice, are relatively few. Notably, by combining maize anther RNA-seq and comparative genomics analyses, 62 orthologs of GMS genes reported in other plants have been predicted previously in maize, consisting of 19 TFs, 18 lipid metabolic genes, and 37 genes involved in other processes [2]. Among them, 35 genes correspond to 16 gene families, and the number of paralogs in each gene family is between 2 and 4 [2]. Analysis of 16 fully sequenced genomes in plants, such as maize, rice, and wheat, has shown that the paralogous gene families account for about 72% of protein-coding genes [11]. Therefore, it is possible that multiple homologous genes are required for male fertility in plants. To date, few homologous GMS genes have been identified, since it is relatively difficult to simultaneously mutate multiple homologous genes through spontaneous mutation or traditional mutagenesis methods. Whether members of a gene family have functional redundancy in male fertility requires mutating two or more paralogs, and then evaluating their phenotypic effects of single and multiple mutations. Thus, the simultaneous editing of multiplex genes makes it possible to simultaneously modify the multi-DNA sequences and further explore the functional redundancy of multiple genes [12].

Gene-editing technologies can efficiently introduce precise and predictable gene mutations into plants to obtain desired phenotypes [13,14]; the CRISPR/Cas system is the most popular system due to its specificity, simplicity, flexibility, and versatility [15]. Multiplex gene editing can be achieved with Cas9 nuclease through expressing Cas9 along with multiple gRNAs [16], which has been successfully used to dissect the functions of gene family members with redundant functions in plants [17]. Additionally, the CRISPR/Cpf1 system is a natural, multi-unit system, with a simple short direct repeat (DR)-based unit itself. The Cpf1 nuclease has the ability to process its own CRISPR RNA and can be used to simplify multiplexed genome editing [18]. Although the CRISPR/Cpf1 system has been shown to efficiently introduce multi-gene mutations into plants, such as maize, rice, and soybean [19–21], it still needs to be further optimized because its editing efficiency is lower than that of the CRISPR/Cas9 system.

Bread wheat is allohexaploid and consists of three genomes (A-, B-, and D-) that exhibit extensive functional redundancy, which makes it unusually difficult to obtain the recessive nuclear male sterile mutants through traditional mutagenesis. Using the CRISPR/Cas9 system, some homologous GMS genes have been reported in bread wheat, including TaMs45, the ortholog of Ms45 in maize and LAP3 in Arabidopsis [22], and TaNP1, the ortholog of OsNP1 and ZmIPE1 [23]. Both TaMs45 triple mutant and TaNP1 triple mutant display complete male sterility, but one WT copy of TaMs45 or TaNP1 genes is sufficient for maintenance of male fertility. In addition, the CRISPR/Cas9 technology has been successfully utilized to identify homologous TF GMS genes in maize [24]. A ZmGAMYB double mutant displayed complete male sterility, but not for the single-gene mutants. Overall, the studies on the
multiplex homologous GMS gene by the CRISPR/Cas9 system are relatively few in plants, and the corresponding research method requires further development.

Here, we established an efficient method to simultaneously mutate multiplex homologous GMS genes in maize with the multi-gene editing strategy based on CRISPR/Cas9. The creation and selection processes of different-type mutants of ZmTGA9-1/-2/-3, ZmDFR1/2, and ZmACOS5-1/-2 families are described in detail. Finally, by combing genotypic and phenotypic analyses, we elucidated the functions of three multi-gene families on male fertility, which will facilitate understanding the functional redundancy of gene family on anther and pollen development.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Maize inbred line Zheng 58 and hybrid Hi-II seeds were provided by our laboratory. All maize materials were grown in the experimental stations at the University of Science and Technology Beijing under normal cultivation conditions, except maize transgenic plants that were grown in a greenhouse under 16 h of light/8 h of dark at 26 °C.

2.2. Characterization of Mutant Phenotypes

A Canon EOS 700D digital camera (Canon, Tokyo, Japan) and a SZX2-ILLB stereomicroscope (Olympus, Tokyo, Japan) were used to take photographs for tassels and anthers, respectively. A BX-53 microscope (Olympus, Tokyo, Japan) was used to assay its mature pollen grains with 1% I₂-KI solution [25].

2.3. Plasmid Construction

For generating the CRISPR/Cas9-mediated multi-gene mutants, the 19-bp fragment targeting the predictive genes were designed on the CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/ accessed on 20 July 2019) and evaluated on a website (http://www.rgenome.net/cas-offinder/ accessed on 20 July 2019), respectively [26]. The vector pCBC-MT1T2 was used, and two 19-bp fragments were amplified based on the primer pair specific for each gene and introduced into the BsaI-digested pBUE411 vector [27]. The primers are listed in Table S1.

2.4. Maize Genetic Transformation

The recombinant plasmids were used for Agrobacterium tumefaciens-mediated transformation into maize (Hi-II) [27]. The Bar gene was used as a selectable marker for selecting positive transformants by PCR amplification using primers OGF41 and OGF42. The primers are listed in Table S1.

2.5. Genotyping Maize Mutant Plants

The cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from the leaves of maize seedlings [28]. PCR amplifications of relevant regions in transgenic plants were accomplished using the specific primers in Table S1. For genotyping plants in T₀ and F₁ generations, the PCR products of relevant regions were purified and introduced into the pEASY-T5 Zero Cloning vector (TransGen Biotech, Beijing, China) for DNA sequencing. The sequencing chromatograms were meticulously analyzed for exact patterns that might indicate the mutation types, i.e., homozygous, monoallelic, or diallelic mutations. The co-segregating molecular markers were designed according to mutations of target genes (Table S1), as described previously [29]. The PCR products were analyzed in F₂ generation using polyacrylamide gel electrophoresis.

2.6. Statistical Analysis

The segregation of sterility phenotypes was assessed in the F₂ populations. Goodness of fit to theoretical ratios was evaluated using the chi-square (X²) test in accordance with
methods as described previously [30], i.e., $X^2 = 6(|O - E| - 0.5)^2/E$ or $X^2 = 6(O - E)^2/E$, where $E$ and $O$ are the expected and observed frequencies, respectively.

3. Results

3.1. ZmTGA9-1, ZmTGA9-2 and ZmTGA9-3 Display Completely Functional Redundancy to Control Anther and Pollen Development in Maize

AtTGA9 and AtTGA10 are involved in regulating a common set of genes that contribute to tapetum and anther development [31]. ZmTGA9-1 (Zm00001d052543), ZmTGA9-2 (Zm00001d042777), ZmTGA9-3 (Zm00001d012294), ZmbZIP87 (Zm00001d038296), and ZmbZIP109 (Zm00001d023424) are the orthologs of TGA9 in Arabidopsis (Figure S1(A1)). However, only ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3 are expressed during maize anther development based on RNA-seq data of W23 anther (Figure S1(A2)) [32], indicating the potentially critical roles of the three genes in controlling maize male reproduction. Based on co-introduced of two vectors containing Cas9 and sgRNA expression cassettes into maize, our previous research revealed that ZmTGA9 triple mutant displayed complete male sterility, while single and double mutants of ZmTGA9-1/-2/-3 showed normal fertility [10]. However, the co-transformation method influences transformation efficiency and especially increases the complexity of molecular identification. To establish a more efficient method for simultaneously editing homologous GMS genes, in this study, we only transferred one CRISPR/Cas9 editing vector into maize to produce novel knockout-mutants of ZmTGA9-1/-2/-3, which further confirmed the function of the ZmTGA9 family on male fertility.

For simultaneously mutating three homologous genes by one gene-editing construct, target 1 was designed in the ninth exon of ZmTGA9-1, and target 2 was designed in the ninth exons of ZmTGA9-2 and ZmTGA9-3 that have highly similar structures (Figure 1(A1,A2)). One vector containing a Cas9 protein and two sgRNA expression cassettes were constructed (Figure 1(A3)), and then introduced into maize hybrid Hi-II by Agrobacterium-mediated transformation to simultaneously edit the three paralogs. Four positive events were obtained in T0 plants and used for further mutation analysis and phenotype observation. DNA sequencing of three target sites revealed that up to 84% of T0 plants contained mutations in one site at least, while five plants generated from one event were homozygous triple mutants. We identified a homozygous loss-of-function triple mutant of ZmTGA9-1/-2/-3 with frameshift and truncation mutations from the five plants mentioned above (Figure S2A), including 1-bp deletion in ZmTGA9-1, and 1-bp insertion in ZmTGA9-2 and ZmTGA9-3, respectively (Figure 1(A2),B), which is a new allelic mutant differing from ZmTGA9 triple mutants obtained previously [10]. Since maize T0 transgenic plants could not usually produce seeds by self-pollination in the greenhouse, we pollinated the T0 plants with pollen grains from maize inbred line Zheng58, and then harvested F1 seeds that could be divided into Cas9-positive (transgene) and Cas9-negative (non-transgene) segregation. To obtain stable mutants that eliminated the interference of sgRNA and Cas9 protein, we chose Cas9-negative F1 plants having WT genotype from Zheng58 and heritable mutations in three paralogs, to perform self-pollination for generating F2 seeds.

To detect genotypes of the derived ZmTGA9-1/-2/-3-Cas9 F2 plants, co-segregating molecular markers with the primer pairs covering the corresponding mutations in ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3 were firstly developed, respectively (Table S1). Furthermore, the genotypes of 85 F2 individuals were identified using PCR amplification together with polyacrylamide gel electrophoresis (PAGE) and classified to eight types: A_/B_/C_, aa/B_/C_, A_/bb/C_, cc, aa/bb/C_, aa/B_/cc, A_/bb/cc, and aa/bb/cc (Figure 2A, (Tables 1 and S2). A further phenotypic analysis showed that only triple homozygous mutant (aa/bb/cc) had shrunken anthers and failed to produce viable pollen grains (Figure 2B), while three single homozygous mutants (aa/B_/C_, A_/bb/C_, and A_/B_/cc) and three double homozygous mutants (aa/bb/C_, aa/B_/cc, and A_/bb/cc) exhibited normal fertility just like WT (A_/B_C) (Figure 2B), indicating that ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3 are completely functional redundant. The proportions of normal and aborted
pollen grains in WT and single, double, and triple mutants of ZmTGA9-1/-2/-3 further confirmed the function of the ZmTGA9 family on male fertility (Figure S3A). In addition, the F2 plants displayed an approximate ratio of 63:1 for fertility to sterility (Table 1 and Table S2), indicating a recessive three-factor inheritance characteristic of the zmtga9-1/-2/-3 mutant. Taken together, the roles of three homologous ZmTGA9 genes in male fertility were further characterized using the efficient CRISPR/Cas9 system.

Figure 1. The CRISPR/Cas9 mutagenesis of ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3 and the derived mutant sequencing analysis. (A) The CRISPR/Cas9 mutagenesis of ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3. (A1) Diagram showing the two target sites on the ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3, respectively. (A2) Sequence analysis of the target sites on ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3 and their mutants. The target sites and protospacer-adjacent motif (PAM) sequences are shown in the antisense strand and highlighted in red and boldface fonts, respectively. Deletions and insertions are indicated by red, short, dashed lines and green fonts, respectively. (A3) Diagram illustrating the sgRNA
expression cassettes targeting ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3 via the dual-sgRNAs CRISPR/Cas9 vector system. (B) Identification of mutations in a homozygous zmtga9-1/-2/-3 triple knockout line by Sanger sequencing. (B1) Sanger sequencing result of mutation in the ZmTGA9-1 gene. (B2) Sanger sequencing result of mutation in the ZmTGA9-2 gene. (B3) Sanger sequencing result of mutation in the ZmTGA9-3 gene. Deletions and insertions are indicated by red, short, dashed lines and green fonts, respectively.

**Figure 2.** Co-segregating molecular marker genotyping and phenotypic characterization of ZmTGA9-1/-2/-3-Cas9 mutants in F$_2$ population. (A) The genotypic identifications of representative ZmTGA9-1/-2/-3-Cas9 F$_2$ individuals using developed co-segregating molecular markers. Molecular markers covering 1-bp deletion, 1-bp insertion and 1-bp insertion in the ZmTGA9-1/-2/-3 genes were detected by using PCR amplification with PAGE, respectively. M, marker. (B) Phenotypic analysis of tassels, anthers and pollen grains stained with 1% I$_2$-KI solution in WT and the single-, double- and triple-
gene homozygous mutants of ZmTGA9-1/-2/-3. A and a, B and b, and C and c represent the WT and mutant genotypes of ZmTGA9-1/-2/-3, respectively. A_/_C_ consists of AA/BB/CC, Aa/BB/CC, AA/Bb/CC, AA/Bb/Cc, AA/Bb/Cc, AA/Bb/Cc, and aa/Bb/Cc genotypes. aa/B_/C_ consists of aa/BB/CC, aa/BB/CC, aa/BB/CC, and aa/BB/CC genotypes. A_/_B_/C_ consists of AA/BB/CC, AA/BB/CC, AA/BB/CC, and AA/BB/CC genotypes. A_/_B_/cc consists of AA/BB/cc, AA/BB/cc, AA/BB/cc, and AA/BB/cc genotypes. A_/bb/C_ consists of AA/bb/CC and AA/bb/CC genotypes.

### Table 1. Genotypic and phenotypic analyses of three knockout lines with simultaneous mutations of two or three homologous genes in F$_2$ generation.

| F$_2$ Lines | Mutation Types | F$_2$ Plants | Expected Ratio | Observed Ratio | $\chi^2$ |
|-------------|----------------|--------------|----------------|---------------|---------|
| ZmTGA9-1/-2/-3-Cas9 | (tga9-1 (aa): −1 bp) / (tga9-2 (bb): +1 bp) / (tga9-3 (cc): +1 bp) | Genotypes A_/B_/C_ aa/B_/C_ A_/bb/C_ A_/B_/cc aa/bb/C_ aa/B_/cc A_/bb/cc aa/bb/cc | 27:9:9:3:3:3:3:1 | 36:15:15:8:2:3:5:1 | 4.43 |
| ZmDFR1/2-Cas9 | (dfr1 (aa): −1 bp) / (dfr2 (bb): −1 bp) | Genotypes A_/B_ aa/B_ A_/bb aa/bb | 9:3:3:1 | 11:5:5:1 | 3.36 |
| ZmACOS5-1/-2-Cas9 | (acos5-1 (aa): +2 bp) / (acos5-2 (bb): +1 bp) | Genotypes A_/B_ aa/B_ A_/bb aa/bb | 9:3:3:1 | 13:6:5:1 | 4.3 |

3.2. ZmDFR1 and ZmDFR2 Have Partially Redundant Functions in Controlling Maize Male Fertility

ZmDFR1 (Zm00001d031488) and ZmDFR2 (Zm00001d020970) are the orthologs of GMS genes OsTKPR1 and AtTKPR1 (Figure S1) [33,34], and the two genes are all preferentially expressed at anther developmental stages S8 to S9 (Figure S1(B2)). To explore their functions on male fertility in maize, we produced both single- and double-gene mutants of ZmDFR1/2 via the CRISPR/Cas9 system.

As ZmDFR has two paralogs in the maize genome (Figure S1(B1,B3)), target 1 was designed in the first exon of ZmDFR1 and target 2 was designed in the first exon of ZmDFR2 (Figure 3(A1,A2)), respectively. One vector simultaneously editing the two paralogs was constructed and transformed into maize immature embryos (Figure 3(A3)), and then two positive events were produced from 94 embryos. To identify the mutation sites of ZmDFR1/2 in primary T$_0$ transgenic plants, PCR products containing the target site fragment were subjected to DNA sequencing. Mutation analysis showed that up to 95% targeted deletions occurred in 14 T$_0$ plants from two events on the target sites of ZmDFR1 and ZmDFR2, and double homozygous mutations were observed in 3 T$_0$ plants from one event. One double homozygous mutant of ZmDFR1/2 with 1-bp deletion in ZmDFR1 and ZmDFR2 (Figure 3(A2),B), respectively, was obtained from the T$_0$ plants. Accordingly, the protein sequences of ZmDFR1 and ZmDFR2 displayed frameshift and truncation mutations (Figure S2B). Similarly, Zheng58 was used as male parent to pollinate the T$_0$ plants of ZmDFR1/2-Cas9 mutant to produce F$_1$ generation, and then Cas9-negative F$_1$ plants were selected to perform self-pollination to further generate F$_2$ seeds.
Figure 3. The CRISPR/Cas9 mutagenesis of ZmDFR1 and ZmDFR2, and co-segregating molecular marker genotyping and phenotypic characterization of ZmDFR1/2-Cas9 mutants in F$_2$ population. (A) The CRISPR/Cas9 mutagenesis of ZmDFR1 and ZmDFR2. (A1) Diagram showing the two target sites on the ZmDFR1 and ZmDFR2 genes, respectively. (A2) Sequence analysis of the target sites in ZmDFR1 and ZmDFR2 and their mutants. The target sites and PAM sequences are shown in the sense strand and highlighted in red and boldface fonts, respectively. Deletions are indicated by red, short, dashed lines. (A3) Diagram illustrating the sgRNA expression cassettes targeting ZmDFR1 and ZmDFR2 genes via the dual-sgRNAs CRISPR/Cas9 vector system. (B) Identification of mutations in a homozygous zmdfr1/2 double knockout line by Sanger sequencing. (B1) Sanger sequencing result of mutation in ZmDFR1 gene. (B2) Sanger sequencing result of mutation in the ZmDFR2 gene. Deletions are indicated by red, short, dashed lines. (C) The genotypic identifications of representative ZmDFR1/2-Cas9 F$_2$ individuals using developed co-segregating molecular markers. Molecular
markers covering 1-bp deletion and 1-bp deletion in ZmDFR1 and ZmDFR2 genes were detected by using PCR amplification with PAGE, respectively. M, marker. (D) Phenotypic characterization of ZmDFR1/2-Cas9 mutants in F2 population. (D1) Phenotypic analysis of tassels, anthers and pollen grains stained with 1% I₂-KI solution in WT and the single- and double-gene homozygous mutants of ZmDFR1/2. (D2) The proportions of normal and aborted pollen grains measured by staining with 1% I₂-KI solution in WT and the single-gene mutants of ZmDFR1 and ZmDFR2 at stage 13 (n = 4220 to 4300). A and a represent the WT and mutant genotypes of ZmDFR1, respectively. B and b represent the WT and mutant genotypes of ZmDFR2, respectively. A_/B_ consists of AA/BB, Aa/BB, AA/Bb, and Aa/Bb genotypes. aa/B_ consists of aa/BB and aa/Bb genotypes. A_/bb consists of AA/bb and Aa/bb genotypes.

To facilitate genotyping F2 progenies, we developed co-segregating molecular markers with the primer pairs covering the mutation fragments in ZmDFR1 and ZmDFR2 (Table S1). The larger and smaller DNA fragment deletions can be detected by PCR amplification together with PAGE, respectively (Figure 3C). Total 156 F2 plants were genotyped, including four genotypes: A_/B_, aa/B_, A_/bb and, aa/bb (Figure 3C, Tables 1 and S3). Phenotypic observation indicated that two single homozygous mutants (aa/B_ and A_/bb) have exerted anthers and display slightly partial male sterility with 10.25% and 32.50% of aborted pollen grains, respectively (Figure 3(D1,D2)), while the double homozygous mutant (aa/bb) exhibits complete male sterility with smaller anthers and without visible pollen grains compared with WT (A_/B_) (Figure 3D). These results showed that the single-gene mutants of ZmDFR1 and ZmDFR2 are partially sterile phenotype, while the ZmDFR1/2 double mutant is a completely male sterile phenotype, indicating ZmDFR1 and ZmDFR2 act partially redundant in controlling pollen development and male fertility in maize. The segregation of fertile to completely sterile individuals in the F2 population fitted an approximate ratio of 15:1. Thus, the male sterility in zmdfr1/2 preserved a recessive dual-factor inheritance.

3.3. ZmACOS5-1 and ZmACOS5-2 Display No Functional Redundancy in Maize Male Fertility

ZmACOS5-1 (Zm00001eb083110) and ZmACOS5-2 (Zm00001eb119050) are the orthologs of GMS genes AtACOS5 and OsACOS12 that play an important role in pollen formation and anther development (Figure S1) [35,36]. In addition, both ZmACOS5-1 and ZmACOS5-2 are highly expressed at maize anther developmental stages S8 and S9 (Figure S1(C2)). The roles of ZmACOS5-1/-2 in controlling male sterility remain unclear in maize. Therefore, we generated a double-gene mutant of ZmACOS5-1/-2 via the CRISPR/Cas9 system to unveil their functions on maize male fertility.

Due to the high sequence similarity in the first exon of ZmACOS5-1 and ZmACOS5-2, two editing targets were designed in the region simultaneously (Figure 4(A1,A2)). One vector containing the two targets was transformed into maize mediated by Agrobacterium. After stable transformation of 102 maize immature embryos, DNAs were extracted from young leaves of 21 T0 plants produced from three positive events to assess the type and frequency of the mutations. Based on PCR amplification and Sanger sequencing of the target sites, at least 17 plants were detected to have mutations at the designed target 1 or target 2, and the same double-homozygous mutations were observed in 5 T0 plants from two events. The double homozygous mutant of ZmACOS5-1/-2 conferred 2-bp insertion in ZmACOS5-5 and 1-bp insertion in ZmACOS5-2, respectively (Figure 4(A2),B), which resulted in a frameshift and truncation mutations of proteins (Figure S2C). Zheng58 was used as a male line to cross with the zmacos5-1/-2 mutant to produce F1 individuals. To dissect the role of a single gene in male sterility, the F2 population was constructed by self-pollination of the Cas9-negative F1 plants.
Figure 4. The CRISPR/Cas9 mutagenesis of ZmACOS5-1 and ZmACOS5-2, and co-segregating molecular marker genotyping and phenotypic characterization of ZmACOS5-1/2-Cas9 mutants in F$_2$ population. (A) The CRISPR/Cas9 mutagenesis of ZmACOS5-1 and ZmACOS5-2. (A1) Diagram showing the two target sites on the ZmACOS5-1 and ZmACOS5-2 genes, respectively. (A2) Sequence analysis of the target sites in on ZmACOS5-1 and ZmACOS5-2 and their mutants. The target sites and PAM sequences are shown in the sense strand and highlighted in red and boldface fonts, respectively. Insertions are indicated by green fonts. The sequence gap length is shown in the middle of the sequences. (A3) Diagram illustrating the sgRNA expression cassettes targeting ZmACOS5-1 and ZmACOS5-2 genes via the dual-sgRNAs CRISPR/Cas9 vector system. (B) Identification of mutations in a homozygous zmacos5-1/-2 double knockout line by Sanger sequencing. (B1) Sanger sequencing result of mutation in the ZmACOS5-1 gene. (B2) Sanger sequencing result of mutation in the ZmACOS5-2 gene.
ZmACOS5-2 gene. Deletions are indicated by red, short, dashed lines. (C) The genotypic identifications of representative ZmACOS5-1/-2-Cas9 F2 individuals using developed co-segregating molecular markers. Molecular markers covering 1-bp insertion and 1-bp insertion in ZmACOS5-1 and ZmACOS5-2 genes were detected by using PCR amplification with PAGE, respectively. (D) Phenotypic analysis of tassels, anthers and pollen grains stained with 1% I2-KI solution in WT and the single- and double-gene homozygous mutants of ZmACOS5-1/-2. A and a represent the WT and mutant genotypes of ZmACOS5-1, respectively. B and b represent the WT and mutant genotypes of ZmACOS5-2, respectively. A_//B_ consists of AA/BB, Aa/BB, AA/Bb, and Aa/Bb genotypes. Aa/B_ consists of aa/BB and aa/Bb genotypes. A_/bb consists of AA/bb and Aa/bb genotypes.

Furthermore, we developed two co-segregating molecular markers with the primer pairs covering mutation fragments in ZmACOS5-1 (with 1-bp insertion) and ZmACOS5-2 (with 1-bp insertion), respectively, which were efficiently used to determine the genotypes of 147 F2 individuals with PAGE (Figure 4C, Tables 1 and S4). In the F2 population, genotypes consisted of A_//B_, aa//B_, A_//bb_, and aa//bb. The corresponding phenotypic analysis showed that the single homozygous zmacos5-1 (aa//B_) exhibits normal anther and pollen development, and the proportion of normal pollen grains is just like WT (Figure S3B). In contrast, the single homozygous zmacos5-2 (A_//bb) and double homozygous zmacos5-1/-2 (aa//bb) display complete male sterility without pollen grains (Figure 4D). All F1 plants from crosses between WT and the double-gene mutant of ZmACOS5-1/2 showed normal fertility, and the resulting F2 plants exhibited a segregation ratio of approximately 3:1 for fertility to sterility, indicating that zmacos5-1/-2 with complete male sterility is caused by a single recessive mutation of ZmACOS5-2. Our results show that ZmACOS5-1 and ZmACOS5-2 are not functionally redundant on male fertility in maize, and ZmACOS5-2 is a GMS gene required for anther and pollen development.

4. Discussion

GMS is generally due to a mutation in a nuclear gene; thus, the GMS mutant has obvious advantages, such as non-conditional, recessive, and strictly monogenic inheritance, and is useful for both hybrid breeding and seed production in crops [37]. Although GMS has long been a research focus for many plants, previous studies of the GMS genes have mainly focused on the single gene [25,38–40]. The main reason is that it is relatively difficult to simultaneously obtain multi-gene mutations with traditional mutagenesis and map-based cloning methods. In fact, analysis of many plant genomes shows that 72% of protein-coding genes are classified into paralogs [11], suggesting many GMS genes containing multiple homologs are unfortunately missed. Therefore, developing the method of multi-gene precise editing is very important for exploring and discovering more GMS genes with functional redundancy.

The CRISPR/Cas9 system, as a highly efficient and convenient genome editing tool, has been successfully used for simultaneously mutating homologous GMS genes in crops, including bread wheat [22,23], maize [24], and cotton [41]. The corresponding GMS genes include TaMs45-A/B/D, TaMsNP1-A/B/D, ZmGAMYB-1/-2, GhGAPAT12/25, etc., which is much less than a single GMS gene. To accelerate the discovery of the homologous GMS genes in maize, we produced mutations in each gene of ZmTGA9-1/-2/-3, ZmDFR1/2, and ZmACOS5-1/-2 families using two gRNA methods in the CRISPR/Cas9 system (Figures 1, 3 and 4). The triple homozygous mutant, zmtdfr1/-2/-3, and double homozygous mutants, zmtdfr1/2 and zmacos5-1/-2, all displayed completely male sterility (Figures 2–4). The Cas9-free F1 plants were selected to generate F2 progenies by self-pollination, and then the F2 progenies were genotyped by specific, developed molecular markers. Combing the genotyping and phenotyping results, the functions of every member in ZmTGA9-1/-2/-3, ZmDFR1/2, and ZmACOS5-1/-2 families on pollen development and male fertility were efficiently defined (Figures 2–4 and Tables S2–S4). Our results confirm the powerful capability of the CRISPR/Cas9 system in inducing multiplex mutagenesis simultaneously, further broadening its application in maize functional genomic research. The CRISPR/Cas9 system
is a convenient tool to study all members of a GMS gene family, which can avoid combining mutations identified in different homologs through conventional crossing.

In addition, in this study, we only used one gene-editing vector to perform the simultaneous mutation of ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3, which was obviously better than the previously reported co-transformation method of two vectors [42]. During maize transformation, the probability of two T-DNA fragments entering an embryonic cell is generally much lower than that of one T-DNA fragment [42]. Therefore, more embryos, reagents, and drugs are demanded for subsequent processes of genetic transformation. On the other hand, more primers need to be designed for genotyping T0 plants; thus, co-transformation takes more time to detect the mutations. Taken together, using the single vector, having dual targets to knock out three ZmTGA9 simultaneously saves both time and labor.

Different GMS genes with multiple homologs differ in functional redundancy. As transcription factor genes, ZmTGA9-1, ZmTGA9-2, and ZmTGA9-3 showed completely redundant functions on the development of anther and pollen. Single and double homozygous mutants in any of the three homologs did not affect pollen development and male fertility, while ZmTGA9-1/-2/-3 triple mutations resulted in completely male sterility without visible pollen grains (Figure 2, Table S2). Both ZmDFR1/2 and ZmACOS5-1/-2 are lipid metabolic genes, and their orthologs in Arabidopsis and rice participate in the sporopollenin metabolon [33,34]. Single-gene mutants of ZmDFR1/2 exhibited slightly partial male-sterility phenotype with exerted anthers, while the double-gene mutant showed complete male sterility (Figure 3, Table S3), suggesting ZmDFR1 and ZmDFR2 are partially functional redundant. The knockout mutant of ZmACOS5-2 showed complete male sterility, while ZmACOS5-1 displayed normal male fertility (Figure 4, Table S4), indicating that they have no functional redundancy in pollen and anther development. The diversified functions of homologs may be induced by the evolution of these gene families. The detailed molecular regulatory pathways of these newly discovered GMS genes contributing to pollen and anther development in maize need further investigations. Furthermore, the biosynthetic pathways of anther cutin, wax, and sporopollenin is relatively conserved in plants [34,43]. Accordingly, many orthologous lipid metabolic GMS genes show highly conserved functions in anther and pollen development between monocots and dicots [44]. Among the lipid metabolic GMS genes identified previously, five were shared among maize, rice, and Arabidopsis, and ten were shared between rice and Arabidopsis [8]. Here, two types (three genes, i.e., ZmDFR1/2 and ZmACOS5-2) of lipid metabolic GMS genes in maize, similar to their orthologs (i.e., AtTKPR1/OsTKPR1 and AtACOS5/OsACOS12) in Arabidopsis and rice, were found to play relatively conserved roles in controlling anther/pollen development and male fertility. These results support the functional conservation of lipid metabolic GMS genes among different plants.

Collectively, using the reverse genetic strategy (e.g., CRISPR/Cas9) to knock out these candidate homologous GMS genes will enrich the maize GMS mutant and gene resources and, thus, facilitate our understanding of the molecular mechanisms underlying pollen and anther development in maize.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11030439/s1, Figure S1. Phylogenetic analysis, expression patterns, amino acid sequence alignment of ZmTGA9-1/-2/-3, ZmDFR1/2 and ZmACOS5-1/-2 families and their putative orthologues or paralogues; Figure S2. Alignment of the amino acid sequences of ZmTGA9-1/-2/-3, ZmDFR1/2, ZmACOS5-1/-2, and their corresponding mutants. Only the sequences flanking the mutations are shown; Figure S3. Pollen characteristics analysis of ZmTGA9-1/-2/-3 and ZmACOS5-1/-2 mutants; Table S1: Primers used in this study; Table S2: Genotypes and phenotypes of all F2 plants derived from ZmTGA9-1/-2/-3-Cas9 line; Table S3: Genotypes and phenotypes of all F2 plants derived from ZmDFR1/2-Cas9 line; Table S4: Genotypes and phenotypes of all F2 plants derived from ZmACOS5-1/-2-Cas9 line.
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**Abbreviations**

- Cas: CRISPR-associated protein
- CRISPR: clustered regularly interspaced short palindromic repeats
- GMS: genic male sterility
- PAGE: polyacrylamide gel electrophoresis
- TFs: transcription factors
- WT: wild type

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