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Pollen-Specific CRISPR/Cas9 System to Increase Heritable Gene Mutations in Maize

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Abstract: The CRISPR/Cas9 system has been widely utilized in plant biotechnology as a gene editing tool. However, a conventional design with ubiquitously expressed CRISPR/Cas9 was observed to cause large numbers of somatic mutations that complicated the identification of heritable mutations. We constructed a pollen-specific CRISPR/Cas9 (PSC) system using pollen-specific promoters of maize *Profilin 1* and *Profilin 3* (pZmPRO1 and pZmPRO3) to drive *Cas9* expression, and the bZIP transcription factor *Opaque2* (*O2*) was employed as the target gene. The maize ubiquitin promoter (pZmUbi)-driven CRISPR/Cas9 (UC) system was employed as a control. We generated transgenic plants for the PSC and UC systems and analyzed three independent events for each system. We found that the pZmPRO1 PSC system generated no target gene mutations in the T0 generation but successfully generated 0–90% target gene mutations in the T1 generation. A total of 31 of 33 mutations in the T1 generation could be inherited in the T2 generation. In addition, 88.9–97.3% of T2 mutations were from the T1 generation. The UC system generated mutations in the T0 generation, and 0%, 50% and 92.9% of T1 mutations were from the T0 generation. Our results demonstrate that the PSC system provided stable, heritable mutants in the next generation, and this approach might also be applied in other crops using germinal cell-specific CRISPR/Cas9 systems to facilitate plant breeding.

Keywords: CRISPR/Cas9; pollen-specific; heritable gene mutation; maize

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1. Introduction

The clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9) system is a form of prokaryotic adaptive immune response. Cas9 and guide RNAs (gRNAs) form a complex that recognizes a specific DNA sequence consisting of a base-pairing sequence to the spacer region of the gRNA and a protospacer adjacent motif (PAM), and this causes a DNA double-strand break (DSB) in the target site [1–3]. The CRISPR/Cas9 system has been demonstrated to be a powerful and efficient genome editing tool and has been widely utilized in a variety of organisms and cell types [4–10]. In plants, the CRISPR/Cas9 system has been applied in many species such as Arabidopsis thaliana [11], rice [12,13], wheat [14], tomato [15] and maize [16–18].

Precise and heritable genome modification is very important in crop breeding. A number of crop traits such as disease resistance [19], flower color regulation [20], herbicide resistance [21] and stress resistance [22] can be improved by the CRISPR/Cas9 system. Normally, strong constitutive promoters are used to achieve a high expression of the

CRISPR/Cas9 system in plants [23–25]. Many target gene mutations are generated. However, at the same time, a large number of somatic mutations are caused, which complicates the identification of heritable mutations and may affect plant growth [12,26].

Maize, as one of the most indispensable crops in the world, feeds a large number of people and plays an essential role in plant research. The first report using the CRISPR/Cas9 system to cause target gene mutations in maize was published in 2014 [16]. The expression of Cas9 is driven by the maize ubiquitin promoter (pZmUbi) in most studies in maize [24,27]. However, with this promoter, the production of somatic mutations is inevitable [28]. A viable strategy to increase heritable gene mutations is to only produce mutations in germ cells. Expression of Cas9 driven by egg cell, pollen or meiosis promoters, which can increase the frequency of heritable mutations and reduce the rate of somatic mutations, has proven to be a feasible approach in plants [29–32]. The haploid induction (HI) system and haploid inducer-mediated genome editing (IMGE) system can accelerate the breeding of various crops [33,34]. The pollen-specific CRISPR/Cas9 system might generate pollen of maize haploid inducer lines to deliver CRISPR/Cas9 reagents into female gametophytes of different maize lines. However, an egg cell- or pollen-specific promoter-driven CRISPR/Cas9 system has not been reported in maize.

In this study, we selected the maize promoters *Profilin 1* (pZmPRO1) and *Profilin 3* (pZmPRO3) to design a pollen-specific CRISPR/Cas9 (PSC) system to improve the production of heritable mutations. A maize pZmUbi-driven CRISPR/Cas9 (UC) system was also constructed as a control. Our results show that the PSC system can increase the rate of heritable mutations and reduce the production of somatic mutations.

2. Materials and Methods

2.1. Plant Materials and Growing Environment

The maize parental line Hi-II was obtained from the Maize Genetics Cooperation Stock Center, maintained in cold storage and grown in the experimental fields. The transgenic plants and WT (wild type) (W22 or B73) were cultivated in a greenhouse at 24–30 °C with a 14 h light/10 h darkness photoperiod at Shanghai University.

2.2. Transformation Vectors Construction

To build the UC system vector, gRNA designed for O2 was synthesized by Generey (Generey.com, Shanghai, China) and cloned into the *Bss*HII site between pU6 and tU6 using gene-specific primers (Table S1). Then, the gRNA cassette was cloned into the *Hin*dIII site of the pCAMBIA3301 vector with the maize codon-optimized Cas9 gene (from Jinsheng Lai's lab) [28]. In addition, Cas9 was driven by pZmUbi.

To construct the PSC system vector, promoter fragments pZmPRO1 and pZmPRO3 were amplified using gene-specific primers (Table S1) from the WT (B73). pZmPRO1 was cloned into the *Hin*dIII site and the *Bgl*II site in the pZmUbi vector of the pCAMBIA3301 vector instead of pZmUbi. Unlike the pZmPRO1 PSC system vector, pZmPRO3 was cloned into the *Pst*I site and the *Bgl*II sites in the pZmUbi vector of the pCAMBIA3301 vector instead of pZmUbi. The gRNA cassette was recombined with the vector by the *Pst*I site using recombinant primers (Table S1).

2.3. Agrobacterium-Mediated Maize Immature Embryo Transformation

Agrobacterium-mediated maize immature embryo transformation was conducted on the basis of Frame et al. [35]. Positive transgenic lines were detected by using bar-specific primers (Table S1).

2.4. Detection of Target Gene Mutations

Maize genomic DNA was extracted with the hexadecyl trimethylammonium bromide method from endosperm or leaves [36]. Specific primer pairs flanking the target gene sites (Table S1) were designed to amplify the target region using Taq 2X Master Mix (plus dye) (Vazyme, Shanghai, China). Of these primers, O2-F1-3/R1-3 were for target1—3,

and O2F4/R4 were for target4. The amplified PCR products were sent for sequencing. Double-peak PCR samples were cloned into the pGEM-T Easy Vector (Promega, Shanghai, China) for DNA sequencing.

2.5. RNA Extraction and Quantitative PCR

Total RNA was extracted using an RNA extraction kit from TIANGEN BIOTECH (Beijing, China), and reverse transcription was also performed using a kit (TOYOBO, Shanghai, China). Fluorescent quantitative PCR (qPCR) was performed using cDNA, PCR-specific primers (Table S1) and SYBR Green qPCR Master Mix (TOYOBO, Shanghai, China).

2.6. High-Throughput Tracking of Mutations

Sequence mutations were also evaluated on the basis of the high-throughput tracking of mutations (Hi-TOM), which is an online tool to track mutations with precise percentages for multiple samples and multiple target sites [37]. Specific primer pairs flanking the target gene sites were designed (Table S1).

3. Results

3.1. Selection and Validation of Pollen-Specific Promoters

To construct the pollen-specific CRISPR/Cas9 (PSC) system in maize, the selection of pollen-specific promoters is crucially important. ZmPRO1–ZmPRO3, as three profilins, were observed to be specifically expressed in male floral tissues in the late stage of pollen development [38,39]. ZmPRO1–3 were first visible in maturing pollen grains (10 mm) and significantly accumulated in anthers dissected from 10 mm spikelets and mature pollen at dehiscence, suggesting that they were anther- or pollen-specific. In this study, we chose the promoters of ZmPRO1 and ZmPRO3 for further investigation. To validate the specific expression of ZmPRO1 and ZmPRO3 in maize, total RNA was extracted from WT (W22) tissues, including the callus, root, stalk, leaf, husk, silk, ear, immature tassel, mature tassel, anther and pollen tissues; the RNA was reverse transcribed and then subjected to qPCR analysis using primers specific to the ZmPRO1 and ZmPRO3 genes (Table S1). Three different biological samples were tested. The results indicate that there were high expression levels of ZmPRO1 and ZmPRO3 only in the anthers and pollen, which is in keeping with the findings of a previous study (Figure 1A,B) [38]. Therefore, we utilized the promoters of ZmPRO1 (pZmPRO1) and ZmPRO3 (pZmPRO3) to drive Cas9 expression for subsequent experiments.

3.2. Design of a Pollen-Specific CRISPR/Cas9 System for Gene Modification

pZmPRO1 and pZmPRO3 were amplified using specific primers (Table S1), which consisted of the 1585 bp sequence upstream of the *ZmPRO1* gene (containing bp 5′UTR) and the 1533 bp sequence upstream of the *ZmPRO3* gene (containing bp 5′UTR), respectively. pZmPRO1, pZmPRO3 and pZmUbi were inserted into the pCAMBIA3301 vector with the maize codon-optimized Cas9 gene (from Jinsheng Lai's lab, Beijing, China) [28] to drive the expression of Cas9 for the pZmPRO1 PSC vector and pZmUbi vector. The bar gene was included in these three vectors as a selectable marker. Opaque2 (O2) is an important transcription factor during maize endosperm development that affects the nutritional value of maize seeds. Therefore, O2 has played an important role in maize research over the past several decades. Accordingly, the O2 protein-coding gene was targeted at four different locations within its coding region according to CRISPR-GE (genome editing) (http://skl.scau.edu.cn/, access on 31 June 2020) [40] in our study (Table S2). The maize U6 promoter (pU6) and U6 terminator (tU6) were amplified using U6-specific primers (Table S1) and cloned to construct the gRNA cassette in the CRISPR/Cas9 vector. Aside from the promoters driving Cas9 expression and enzyme sites, there was no difference among the pZmPRO1 PSC vector, the pZmPRO3 PSC vector and the pZmUbi vector (Figure 1C). The pPSC (pZmPRO1 and pZmPRO3) and UC (pZmUbi) vectors

were constructed. In total, three different vectors were created to perform plant gene modification studies in maize using Agrobacterium-mediated maize immature embryo transformation.

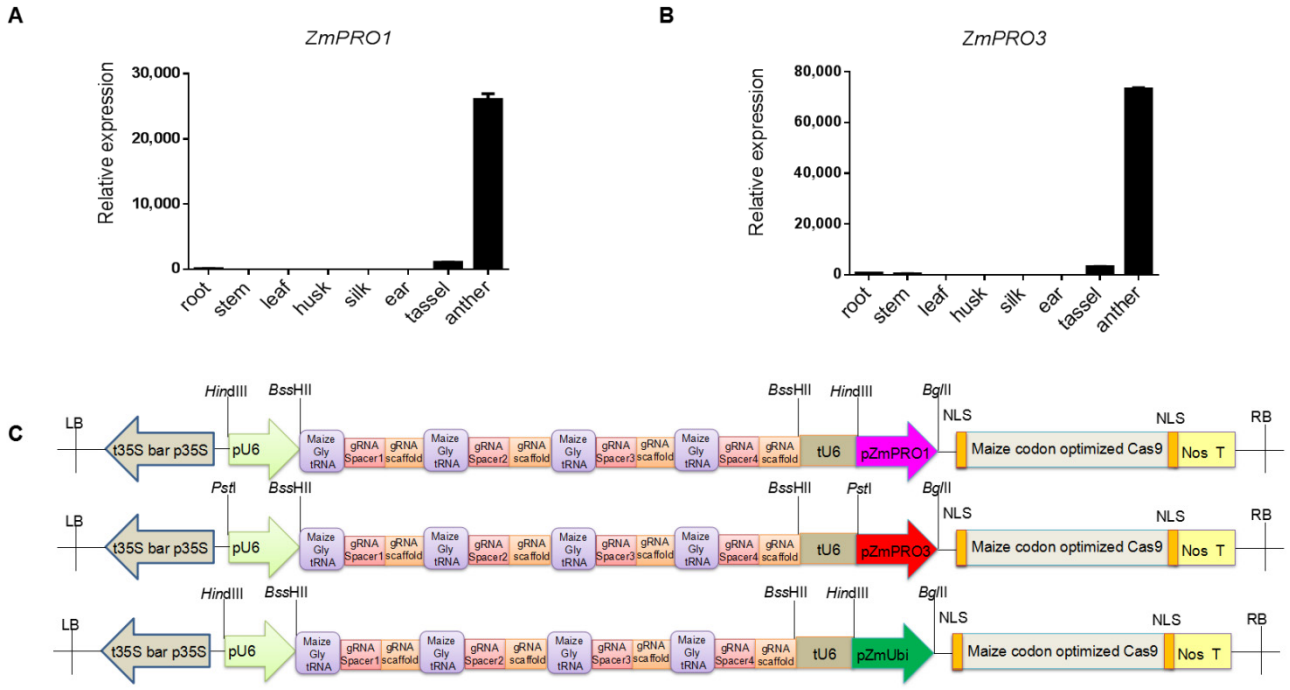

Figure 1. Expression patterns of *ZmPRO1* and *ZmPRO3* genes and component diagram of vector construction. (**A**,**B**) RNA expression levels of *ZmPRO1* and *ZmPRO3* in various tissues in W22. For each RNA sample, three technical replicates were performed. Values are means with SE; $n = 3$ biological replicates. (**C**) Structures of pZmPRO1, pZmPRO3 and pZmUbi driving *Cas9* vector based on pCAMBIA3301. pU6, maize U6 promoter; tU6, maize U6 terminator; pZmPRO1, *ZmPRO1* promoter; pZmPRO3, *ZmPRO3* promoter; pZmUbi, maize ubiquitin promoter; NLS, nuclear localization sequence; Nos T, nopaline synthase terminator; LB, left border; RB, right border; *bar*, herbicide resistance *bar* gene; t35S, 35S terminator; p35S, 35S promoter.

3.3. Acquisition and Analysis of T0 Generation Transgenic Plants

After successful transformation, three individual T0 positive transgenic events were obtained for each vector. They were independent events and derived from individual embryos. Transformed plants were confirmed by PCR amplification of the screening marker gene *bar* with primers bar-F and bar-R (Table S1) (Figure S1). To verify the validity of pZmPRO1 and pZmPRO3, qPCR analysis of *Cas9* expression in the root, stem, leaf, husk, silk, ear, tassel and anther (including pollen) tissues was performed. According to the results of this analysis, the expression level of *Cas9* in the anther tissue was markedly higher than that in other tissues, although there was a minor level of expression in the leaf and tassel tissues of pZmPRO1 PSC plants and in the leaf tissue of pZmPRO3 PSC plants (Figure S2), which suggests that the promoter fragments were effective.

Genomic DNA was extracted from every T0 transgenic seedling. Sequencing for a 1561 bp fragment amplified by O2-F/R containing target1-4 was observed to fail frequently due to signal disruption. Therefore, an 847 bp fragment flanking target1-3 and a 329 bp fragment containing target4 were amplified by O2-F1-3/R1-3 and O2-F4/R4 (Table S1), specifically for DNA sequencing. Different mutations were found in every T0 transgenic plant generated by the UC system, which probably contained somatic mutations. Among these mutations, there were different mutations at target1 and the same mutations at target4. There was no mutation at target2 and target3, except for a 20 bp deletion in the #2 mutant line of the UC system. This result demonstrates that our sgRNAs were working. The overall gene mutations caused by the UC system at every target site in the

T0 generation are shown in Figure 2A. Conversely, no target gene mutation was generated by the pZmPRO1 and pZmPRO3 PSC systems in T0 plants. This result confirms that the PSC system was pollen-specific and did not generate mutations during tissue culture or the development of T0 plants.

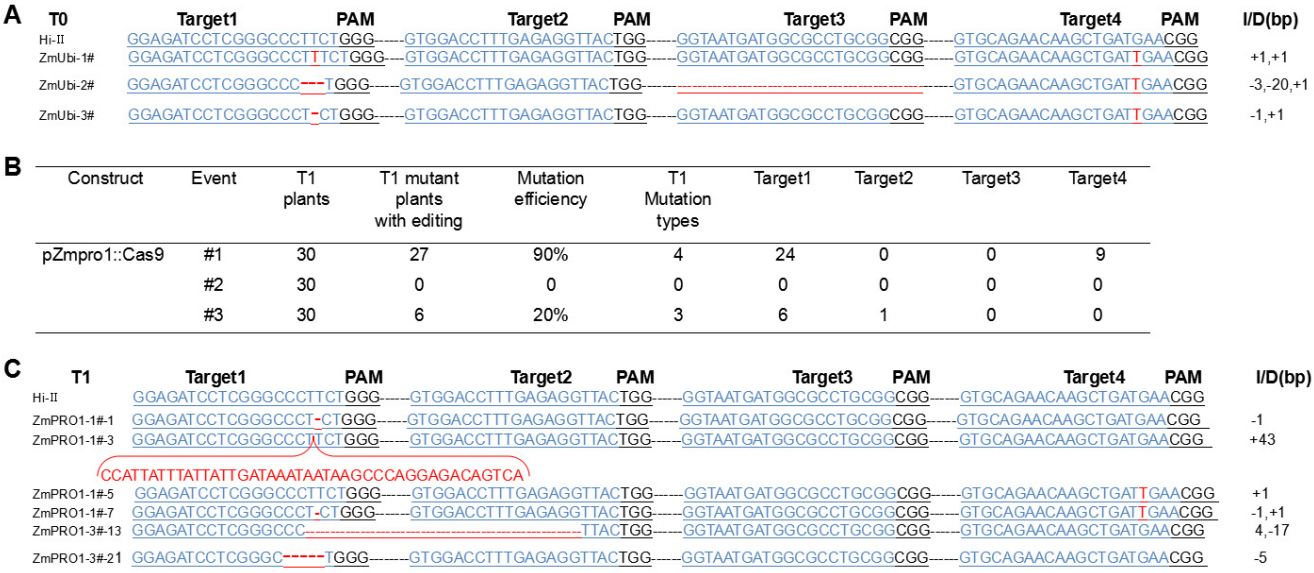

Figure 2. Target gene mutations generated by the UC system in the T0 generation and by the pZmPRO1 PSC system in the T1 generation. (**A**) Target gene mutations generated by the UC system in the T0 generation. The DNA sequences of four targets are provided. The 20 bp gRNA spacer sequence is in blue, and the PAM site is in black. Gene mutations are in red. I/D represents insertions and deletions, respectively. The lengths of insertions and deletions are presented. (**B**) Summary of T1 mutation results of pZmPRO1 PSC system. (**C**) Target gene mutations generated by the pZmPRO1 PSC system in the T1 generation. The DNA sequences of four targets are provided. The 20 bp gRNA spacer sequence is in blue, and the PAM site is in black. Gene mutations are in red. I/D represents insertions and deletions, respectively. The lengths of insertions and deletions are presented.

3.4. PSC System Causes Target Gene Mutations in the T1 Generation

To test the efficiency of the PSC system, all the T0 plants as male parents were subsequently crossed with the WT (W22) in the greenhouse to produce T1 generation lines. T1 seeds from the PSC system were collected and identified. To study, in greater detail, the number and nature of the gene mutations present in the PSC system, thirty individual T1 seeds from each T0 transgenic line were randomly selected. DNAs were prepared from the endosperm of T1 seeds. Additionally, the target regions were PCR amplified and sequenced using the genome DNAs. The results show that target1, target2 and target4 were mutated in the pZmPRO1 PSC system in the T1 generation. There were 27 T1 mutant plants of #1, 0 T1 mutant plants of #2 and 6 T1 mutant plants of #3 in total, and the mutation frequencies were 90%, 0% and 20% in #1, #2 and #3, respectively. Twenty-four plants were mutated at target1, and nine plants were mutated at target4 (Figure 2B). Six types of target gene mutations were observed to be caused by the pZmPRO1 PSC system, including four mutation types in #1 and three mutation types in #3 (Figure 2C). Duplicated mutations were merged. Detailed mutation information for the pZmPRO1 PSC system is presented in Table S3. Our results suggest that the PSC system was effective and could be utilized to generate different mutations in the T1 generation.

Next, we checked plant leaves. In the T1 plants of the pZmPRO1 PSC system, the marker gene *bar* was also detected (Figure S3). Based on the presence of *bar*, line #1 likely had multiple T-DNA loci, and line #3 likely had a single T-DNA locus. All the T1 mutant plants generated by the PSC system were heterozygous (Figure S4). No new target mutation in the leaves was detected in the mutant T1 plants we selected, whose mutations were

the same as those of the mutations we detected in the endosperm. This result confirms that the PSC system promoters only worked during the pollen period and reduced the influence of somatic mutations, and this property contributed to the acquisition of heritable mutants. However, no target mutation was generated by the pZmPRO3 PSC system in the T1 generation.

3.5. PSC System Increases Heritable Mutations and Reduces Somatic Mutations

To compare the heritable gene mutations created by the UC system in the T0 generation, T0 plants were crossed with the WT (W22), and T1 seeds were obtained. For each of the three events generated by the UC system, 15 individuals in the T1 generation were subjected to mutation detection through Sanger sequencing of PCR products. We were looking at inherited mutations that first appeared in T0 pollen and not de novo mutations that arose in the Cas9-positive T1 pollen, and thus we did not split up our T1 plants according to *bar*-positive or negative. The results show that there were 14 (#1), 15 (#2) and 12 (#3) mutated T1 plants in the T1 generation. Of the three mutated T0 plants, two (#1 and #3) T1 lines had heritable mutations from the T0 generation. Of these T1 mutations, 0%, 50% and 92.9% of the mutations generated by the UC system were from the T0 plants (Figure 3A). All the T1 mutations caused by the UC system are shown, in detail, in Table S4. This result suggests that the UC system was efficient in generating mutations in transgenic plants, but, at the same time, a large proportion of somatic mutations were also produced.

A

Construct	Event	No. of tested T1 plants	No. of T1 plant with mutations	T1 plants with mutations	
				% from T0	% New at T1
pZmUbi::Cas9	#1	15	14	92.9	7.1
	#2	15	15	0	100
	#3	15	12	50	50

B

Construct	Event	No. of tested T2 plants	No. of T2 plant with mutations	T2 plants with mutations	
				% from T1	% New at T2
pZmPRO1::Cas9	#1	81	37	97.3	2.7
	#2	10	0	0	0
	#3	18	9	88.9	11.1

Figure 3. Target mutations generated by the UC system and the pZmPRO1 PSC system in different generations. (**A**) Target mutations generated by the UC system in the T1 generation. (**B**) Target mutations generated by the pZmPRO1 PSC system in the T2 generation.

To explore the heritable mutations generated by the pZmPRO1 PSC system, mutated T1 Cas9-positive plants (#1 and #3) and T1 Cas9-positive plants (#2) were self-crossed to produce T2 plants, which were subsequently identified. We were looking at inherited mutations that first appeared in T1 pollen and not de novo mutations that arose in the Cas9-positive T2 pollen, and thus we did not split up our T2 plants according to *bar*-positive or negative. Since the pZmPRO3 PSC system did not create target gene mutations in the T0 and T1 generations, no T2 plants were analyzed. A total of 3 pZmPRO1 PSC system T2 seeds from 27 (#1) and 6 (#3) mutant T1 plants (99 T2 seeds in total) and 10 T2 seeds from 3 Cas9-positive T1 plants of #2 generated by the pZmPRO1 PSC system were selected randomly to verify whether the T1 mutations could be inherited. The results suggest that of the 27 (#1) and 6 (#3) mutated T1 plants, 26 (#1) and 5 (#3) T1 mutations could be inherited in the T2 generation. A total of 88.9–97.3% of the mutations in the T2 generation generated by the pZmPRO1 PSC system were derived from the T1 plants, which was considerably higher than in the UC system (Figure 3). In order to evaluate the genome editing efficiency of some plants verified by Sanger sequencing, the editing efficiency of #3 generated by the UC system in the T0 generation and #3 generated by the pZmPRO1 PSC system in the T1

generation was confirmed based on the high-throughput tracking of mutations (Hi-TOM). The Hi-TOM results are consistent with the editing efficiency based on the PCR and Sanger sequencing data (Table S6). These results demonstrate that the PSC system could improve the production of heritable mutations.

Of these T2 plants from T1 PSC Cas9-positive plants, new target mutations were generated at target1 in #1-10-2 and #3-21-1, which are shown in red in Table S5. The new mutation rate of the pZmPRO1 PSC system was only 0–11.1% (Figure 3B). In addition, no mutation was generated in the T2 plants of #2.

4. Discussion

Highly efficient and accurate heritable gene modifications contribute to plant genetics and breeding. However, the CRISPR/Cas9 system using constitutively strong promoters may generate unwanted somatic mutations in various mutant tissues and growth periods [28]. Although a germinal cell-specific CRISPR/Cas9 system using the promoter of the SPOROCYTELESS gene was determined to be efficient in Arabidopsis thaliana, no homologous gene to the SPOROCYTELESS gene [30] can be found in certain other plants, including maize. To overcome the limitation of the UC system, we developed a pollen-specific CRISPR/Cas9 system to increase heritable mutations and reduce somatic mutations in maize. In our study, the results suggest that the pZmPRO1 PSC system was highly effective in avoiding somatic mutations. This system generated target gene mutations in the T1 generation instead of the T0 generation (Figure 2B,C). Our results indicate that 88.9–97.3% of the mutations were stably inherited from the T1 to T2 generations (Figure 3B). Meanwhile, the mutations showed a higher degree of diversity that benefits the acquisition of various mutants (Figure 2C).

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Unfortunately, no mutation was produced in the T0 and T1 generations by the pZmPRO3 PSC system. This result might be partly attributable to the lower Cas9 expression driven by pZmPRO3 compared to pZmPRO1. The mutation efficiency of the CRISPR/Cas9 system largely correlated with the expression of both Cas9 and sgRNAs [31]. According to the absolute expression value data of ZmPRO1 and ZmPRO3 from maize GDB (https://www.maizegdb.org/, access on 31 January 2021), ZmPRO1 expression is higher than that of ZmPRO3. The tissue specificity of the expression and the level of expression should both be considered when selecting pollen-specific promoters. Adding an enhancer in front of the pollen-specific promoter to increase Cas9 expression without losing the tissue specificity of the expression might also be a useful approach for improving the mutation efficiency of the PSC system.

In the PSC system, almost no target gene mutation was identified in the somatic cells, which substantially reduced the workload necessary to identify mutations and the production of unwanted mutations during the growth and development of plants. The PSC system can also be utilized in the HI-Edit system or IMGE system by delivering CRISPR/Cas9 reagents into female gametophytes of different maize lines which can ac-

celerate crop breeding [33,34]. In the IMGE system, the Cas9 protein could still be present after fertilization from the pollen cell and could still be present in the fertilized egg cell to edit the maternal genome. In addition, there are homologous genes of ZmPRO1 in *Sorghum bicolor* and *Oryza sativa* which suggests that the pZmPRO1-driven CRISPR/Cas9 system may be effective in other crops and may facilitate considerable progress in research on crop genetics and breeding.

5. Conclusions

In conclusion, this is the first report to describe a successful and effective CRISPR/Cas9 system for gene modification in maize pollen cells. This PSC system-based strategy for maize can notably increase the production of heritable mutations and reduce the production of somatic mutations. Further application of the PSC system might contribute to the acquisition of a variety of heritable mutants and facilitate the genetic screening of sterility or other desired mutations in crops.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11080751/s1. Figure S1. Identification of T0 transgenic lines generated by the UC system and the PSC system. Figure S2. Expression patterns of *Cas9* in the PSC system. Figure S3. Identification of T1 transgenic lines generated by the pZmPRO1 PSC system. Figure S4. The sequencing chromatographs of PCR products of #1 generated by the pZmPRO1 PSC system in the T1 generation. Table S1. Primers used in this study. Table S2. Targets in this study (Hi-II). Table S3. Target gene mutations caused by the pZmPRO1 PSC system in the T1 generation. Table S4. Target gene mutations caused by the UC system in the T1 generation. Table S5. Target gene mutations caused by the pZmPRO1 PSC system in the T2 generation. Table S6. Editing efficiency of #3 generated by the UC system in the T1 generation and #3 generated by the pZmPRO1 PSC system in the T2 generation on the basis of Hi-TOM.

Author Contributions: R.S. and W.Q. conceived and designed the research. C.J., M.W. and P.F. performed experiments. C.J. collected and analyzed the data. R.S., W.Q. and C.J. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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