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CRISPR/Cas9-Mediated *BocPDSs* Gene Editing in Chinese Kale Using the Endogenous tRNA-Processing System

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Abstract: Chinese kale is a native vegetable from the *Brassicaceae* family that is grown extensively in Southeast Asia and Southern China. Its low genetic transformation and gene editing efficiency hinder gene function research and molecular biology in Chinese kale. CRISPR/Cas9 is a useful tool for plant genome research due to its rapid development and optimization. This study targeted *BocPDSs*, (*BocPDS1*, *BocPDS2*) to establish an effective CRISPR/Cas9 system in Chinese kale. A tandemly arranged tRNA-sgRNA construct was used to express numerous sgRNAs to induce *BocPDS1* and *BocPDS2* double and single mutations, with a mutation rate of 61.11%. As predicted, several mutant plants showed an albino phenotype with a harbored mutation in an exon and intron region, highlighting the relevance of the intron. The presence of mutations in the intron region suggests that the cleavage process in Chinese kale, utilizing CRISPR/Cas9 shows a preference for AT-rich regions. The distinct and somewhat redundant functions of *BocPDS1* and *BocPDS2* are demonstrated by the complete albino phenotype of the double mutants and the mosaic albino phenotype of the individual *BocPDS1* and *BocPDS2* mutants. Specific gene editing modes, including base deletion, base substitution, and base insertion, were identified in the sequence of the target gene. Among them, short nucleotide insertions were the most common type of insertion, with base insertions having the highest frequency (61.54%). Furthermore, no instances of off-target gene editing were detected. The current work demonstrated that the CRISPR/Cas9 gene editing system, which relies on endogenous tRNA processing, can effectively induce mutagenesis in Chinese kale. This finding establishes a theoretical basis and technical backbone for the more effective implementation of CRISPR/Cas9 gene-editing technology in Chinese kale and Brassica plants.

Keywords: Chinese kale; tRNA-sgRNA; CRISPR/Cas9; *BocPDSs*; mutation

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1. Introduction

Precision genome editing by site-directed mutation technologies can yield high-quality agricultural and other products in substantial volumes, addressing the escalating demands of society [1]. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), gene editing tools utilized for over a decade, are intricate, expensive, and difficult to execute [2,3]. The concurrent publication of two studies in the journal Science showcased the potential of the CRISPR/Cas system for site-specific alteration of human cell genes, thereby affirming its efficacy in eukaryotic genome editing [4,5]. CRISPR/Cas9 is quickly becoming the first choice of researchers and is widely used in various species, including plants, due to its simple expression vector construction, low cost, ability to modify multiple targets at once, and accurate and efficient targeting efficiency. Thus far, it has been successfully used in *Nicotiana benthamiana* [6], *Arabidopsis* [7], wheat [8], rice [9], maize [10], sorghum [11], potato [12], sweet orange [13], tomato [14], grape [15], poplar [16], citrus [17], and apple [18].

CRISPR/Cas9 depends on the complementary base pairing between the target sequence and the sgRNA sequence to direct the Cas9 protein to bind to the target region and cleave DNA, resulting in double-strand breaks (DSBs) [19,20]. Mutations such as gene insertion, deletion, or replacement can be induced by triggering homologous recombination (HR) or non-homologous end joining (NHEJ) at the cell level [21,22]. The independence of each guide RNA (sgRNA) enables CRISPR/Cas9 technology to alter several genes or loci inside a single cell. Nonetheless, the effectiveness of CRISPR/Cas9-mediated editing of many genes and loci is limited and contingent upon the expression levels of sgRNA or Cas9. In further research, scientists combined tRNA and gRNA to construct a polycistronic gene and associated two or more sgRNAs within a single production vector [23]. The generation of a considerable amount of sgRNAs with the precise targeting sequence markedly improves mutation efficiency. This approach has been effectively implemented for rice, corn, wheat, and various other crops [23], which has greatly promoted the genetic research of plants and improved crop varieties.

The CRISPR/Cas9 system is becoming a powerful tool for genome editing in plants [24–26]. However, only a few successful examples of genome editing have been reported in *Brassica* plants [27]. Chinese kale (*Brassica oleracea* var. *chinensis* Lei) is an important *Brassica* leafy vegetable crop and is one of the vegetables unique to South China [28]. It is widely planted in Taiwan, Guangdong, Guangxi, and Fujian, along with the northern agricultural region, where the production of Chinese kale has increased annually [28]. The stem and young leaves of Chinese kale are nutritious and rich in anticancer bioactive compounds and glucosinolates [29].

The *phytoene desaturase* (*PDS*) gene, which encodes a crucial enzyme in carotenoid production, is often employed as the target gene for CRISPR/Cas9 research in plants because the albino phenotype generated by *PDS* disruption is easily identifiable [30]. To facilitate effective mutagenesis in Chinese kale, we developed a CRISPR/Cas9 gene editing technique that makes use of endogenous tRNA processing. This research utilized a tandemly arranged tRNA-sgRNA sequence to make multiple sgRNAs through plant endogenous tRNA processing. The Chinese kale phytoene desaturase gene *BocPDSs* was chosen as the target gene for examining genome-editing efficiency via tRNA processing. The CRISPR/Cas9 system effectively knocked out target genes and induced mutagenesis utilizing the tRNA-processing machinery. Our study shows that the CRISPR/Cas9 system and endogenous tRNA processing can significantly modify Chinese kale genes. This establishes a theoretical basis and technical grounds for the increasingly effective implementation of CRISPR/Cas9 gene-editing technology in Chinese kale.

2. Materials and Methods

2.1. Vector Construction

The pCACas9 and sgRNA vectors were generously provided by the College of Horticulture and Landscape Architecture at Southwest University. A polycistronic tRNA-sgRNA-expressing cassette sequence was synthetically constructed with a "G" nucleotide inserted between the Arabidopsis U6-26 promoter and the initial glycine-tRNA sequence of Arabidopsis to fulfill the transcription initiation criteria of the U6 promoter. Additionally, the sgRNA structure was altered by lengthening the duplex length by ~5 bp and changing the fourth thymine to cytosine in the continuous sequence [27]. Four common target sequences of *BocPDS1* and *BocPDS2* were designed via the online software of CRISPR-GE (http://skl.scau.edu.cn/, accessed on 2 March 2023) [31]. Complementary oligonucleotides of selected loci were integrated into the *Bbs*I, *Bsa*I, *Bsm*BI, and *BfuA*I sites of the tRNA-sgRNA-expressing cassette, resulting in U6-26::tRNA-sgRNA-BocPDS-ABCD-expressing cassettes including a *Bam*HI site at the 5′ end and an EcoRI site at the 3′ end. The U6-26::tRNA-sgRNA-BocPDS-ABCD-expressing cassettes were ligated into the *Bam*HI and *Eco*RI sites of pCACas9 to create the pCACas9-tRNA-sgRNA-BocPDS-ABCD vectors for the transformation of Chinese kale.

2.2. Chinese Kale Transformation

An elite inbred line of Chinese kale 'aijiaoxianggu' was used in this study for gene transformation. After sowing sterilized seeds on seeding medium (1/2 MS, 3% sucrose, and 0.6% agar), they were placed in tissue culture chambers at a temperature of 25/20 °C (day/night), a light cycle of 16/8 h (day/night), and a light intensity of 100 μmol m^{-2} s^{-1}. After six or seven days, the cotyledons and hypocotyls of sterile seedlings were cut, used as explants, and pre-cultured on callus initiation medium (MS, 0.05 mg/L IAA, 2 mg/L TZT, 7 mg/L $AgNO_3$, 3% sucrose, and 0.6% agar) in the dark for 2 days at 25 °C before inoculation and co-culture with *Agrobacterium tumefaciens* EHA105. The preincubated hypocotyls were soaked in *Agrobacterium*-infection buffer (MS, 3% sucrose, and 1 mg/L AS; pH 5.8–6.0) for 10 min and transferred to the co-cultivation media (MS, 0.05 mg/L IAA, 2 mg/L TZT, 7 mg/L $AgNO_3$, 3% sucrose, 0.6% agar, and 1 mg/L AS) in the dark for 2 days at 25 °C. After one week of delayed selection, the explants were transferred to callus- and shoot-induction media (MS, 0.05 mg/L IAA, 2 mg/L TZT, 7 mg/L $AgNO_3$, 3% sucrose, 0.6% agar, 300 mg/L timentin (Tim), and 5 mg/L phosphinothricin (PPT)). The secondary medium was changed every two weeks. When the regenerating shoots reached 3–5 cm in height, they were transferred to rooting media (MS, 0.2 mg/L IAA, 3% sucrose, 0.6% agar, and 300 mg/L Tim) to obtain phosphinothricin-resistant plantlets.

2.3. Transformation Efficiency Detection

A total of roughly 3200 explants were used for infection during the four batches of Chinese kale genetic transformation, and the number of transgenic and mutant plants was tallied. Genomic DNA was isolated from the shoots of phosphinothricin-resistant and wild-type plants utilizing a standard cetyltrimethylammonium bromide (CTAB) protocol. PCR amplification was conducted with carrier-specific primers (Table S1). The PCR products were detected using electrophoresis to confirm the Chinese kale transformants and to calculate transformation efficiency. The calculation formula for the PCR positive rate and the conversion rate is as follows:

$$PCR\ postive\ rate\ (\%) = \frac{The\ number\ of\ resistant\ bud\ or\ plants\ with\ the\ PCR\ amplified\ target\ band}{Number\ of\ resistant\ buds\ or\ plants\ detected\ by\ PCR} \times 100\%$$

$$Conversion\ rate\ (\%) = \frac{Number\ of\ resitant\ plants\ with\ target\ band\ amplified\ by\ PCR}{Total\ number\ of\ explant\ infected} \times 100\%$$

2.4. Mutation Detection

To detect whether the mutation of the *BocPDS1* or *BocPDS2* gene was introduced into transgenic plants. The DNA of transgenic plants was used as a template, and the full length of *BocPDS1* and *BocPDS2* genes in each transformant were amplified using gene-specific primers (Table S1). Following that, the PCR products were sequenced. Sequences were compared by DNAMAN software (version 6.0; Lynnon Corporation, Canada) to calculate mutation rate and analyze mutation types. The calculation formula for the mutation rate is as follows:

$$Mutation\ rate\ (\%) = \frac{The\ number\ of\ transgenic\ plants\ harboured\ mutation}{The\ number\ of\ transgenic\ plants} \times 100\%$$

2.5. Off-Target Analysis

The CRISPR-GE online software (http://skl.scau.edu.cn/, accessed on 2 March 2023) allows users to access key information about a target, including its sequence, position, plus and minus strands, GC content, probable off-target sites, and estimates [31]. The sites with the greatest likelihood of off-target effects were chosen, and the sequences surrounding these sites were amplified using particular primers (Table S1). An analysis of the PCR product was conducted using Sanger sequencing.

3. Results

3.1. SgRNA Design and Vector Construction

Four common target sites were found throughout the exon sequences of *BocPDS1* and *BocPDS2*. Sites A and B were located within the third exon, while sites C and D were positioned within the sixth and seventh exons, respectively (Figure 1A). The target site sequences A, B, C, and D were sequentially integrated into the *BbsI*, *BsaI*, *BsmBI*, and *BfuAI* sites of the synthetic tRNA-sgRNA vector. The sgRNA-expressing cassette was integrated into the pCACas9 vector, yielding the pCACas-tRNA-sgRNA-BocPDS-ABCD vector (Figure 1B).

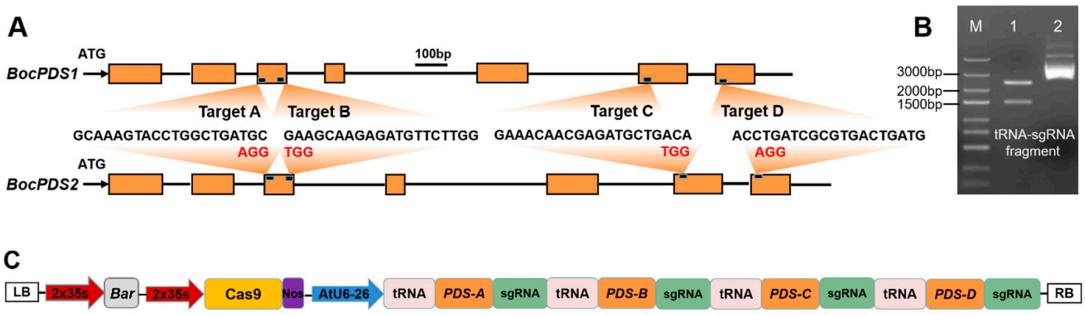

Figure 1. Vector construct for genome editing of the *BocPDSs* genes. (**A**) Illustration depicting the BocPDSs gene segment, highlighting the four designated target locations. The orange boxes represent exons, whereas the black lines highlight introns. The target sequence is displayed in black typography. The PAM is specified in red letters. (**B**) Analysis of the cloned portion of the tRNA-sgRNA expression cassette using gel electrophoresis. Lane 1: plasmid cleaved by PvuI; Lane 2: plasmid genomic DNA. (**C**) This diagram depicts the genetically modified CRISPR/Cas9 vector that incorporates a tRNA-processing system utilizing multiplex sgRNAs for the *BocPDS1* and *BocPDS2* genes.

3.2. CRISPR/Cas9 Transformation Efficiency in Chinese Kale

We employed the established genetic transformation procedure from our laboratory (Figure 2) to perform Agrobacterium-mediated transformation on around 3200 explants. A total of twenty-six plants resistant to phosphinothricin were successfully acquired. The genomic DNA was isolated from the shoots of plants resistant to phosphinothricin and plants of the wild type. Employing carrier-specific primers for PCR amplification verified the presence of the trans-formed constructs in the transgenic lines. All 18 resistant plants effectively amplified a 550 bp target band, whereas the non-template (negative control), wild-type plants (negative control), and the other 8 resistant plants did not exhibit the amplified target PCR band (Figure 2G). Ultimately, the transformation efficiency was determined to be 0.56%, while the PCR-positive rate stood at 69.23%. The presented results demonstrate the successful transfer of the target expression cassette into the 18 Chinese kale lines.

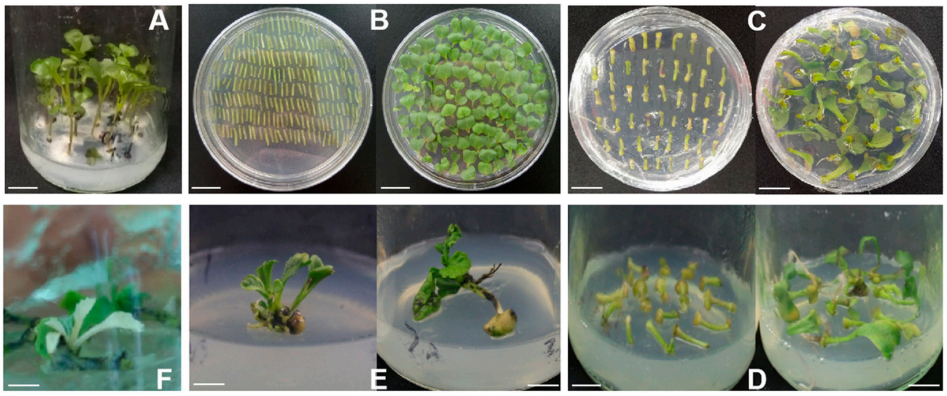

Figure 2. *Cont.*

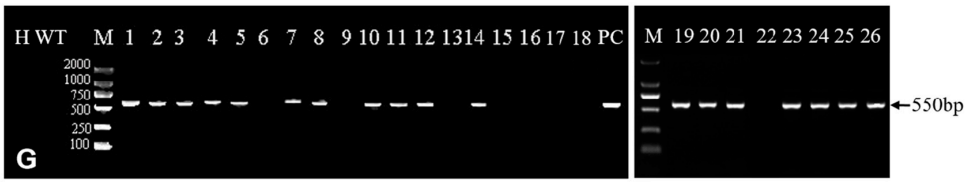

Figure 2. Methodology for Agrobacterium-mediated genetic modification in Chinese kale. The experimental procedures include (**A**) septic seedling culture; (**B**) pre-culture; (**C**) co-culture; (**D**) delayed screening; (**E**) resistance screening; and (**F**) subculture. (**G**) PCR identification of plants resistant to BocPDSs that have been modified using CRISPR/Cas9. M: DNA marker size; plasmid PC, which contained the vector and single guide RNA (sgRNA), served as the positive control. The negative control consisted of H, WT: H_2O, and the DNA of the wild type. WT indicates a non-transforming plant. 1–26: denotes the numerical value of the resistant plant line. An arrow indicates the target band.

3.3. Albino Phenotype of the BocPDSs Mutants

PDS is a key enzyme in the plant carotenoid biosynthesis pathway. Knockout of the *PDS* gene will block the chlorophyll synthesis of plants and lead to an albino phenotype [1]. The 11 *BocPDSs* mutants developed exhibited a distinct albino phenotype while compared to the wild type (Figure 3). This is similar to the knockout phenotype of the *PDS* gene previously reported in rice [2], tobacco [3], petunia [4], poplar [5], apple [6], kiwifruit [7], cabbage [8], and other species, indicating that the gene function of *BocPDSs* had been destroyed, leading to an albino phenotype in gene-edited plants. Within this group, two lines displayed the entirely albino phenotype, while nine lines were chimeras, displaying a mosaic albino phenotype. Remarkably, the foliage of plants carrying both *BocPDS1* and *BocPDS2* double mutations exhibited distinct albino characteristics, both in their pure and mutant forms (Figure 3A–C). Conversely, plants with either *BocPDS1* or *BocPDS2* single mutations displayed a mosaic albino phenotype characteristic of chimeric mutants (Figure 3D–G).

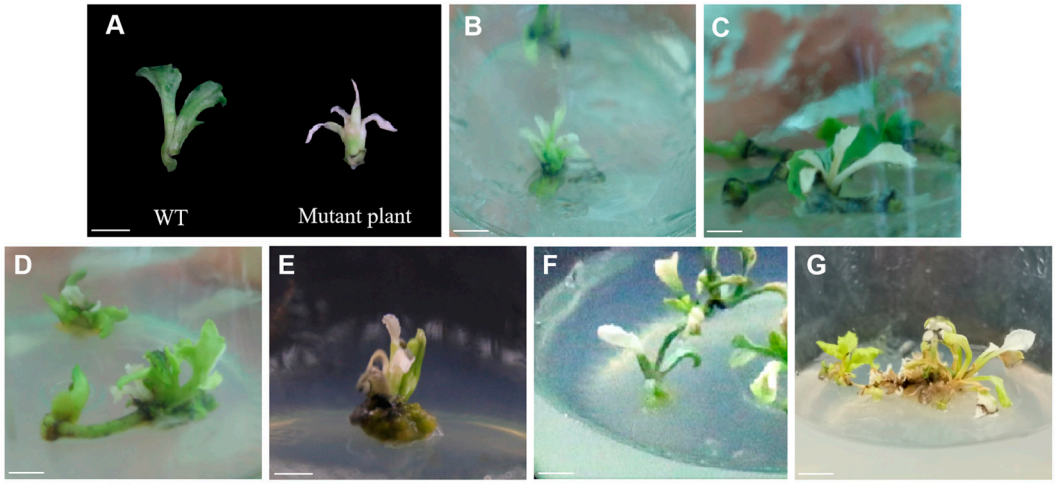

Figure 3. The phenotype of CRISPR/Cas9-edited *BocPDSs*-resistant plants. (**A**) Comparison image of wild-type (non-transforming plant) and mutant plants; (**B**,**C**) pure albino phenotypes; (**D**–**G**) chimeric albino phenotype.

3.4. Analysis of the BocPDSs Mutation Efficiency

A PCR was conducted on 18 transgenic lines using specified primers to amplify the complete length of *BocPDS1* and *BocPDS2* to assess the mutation effectiveness of CRISPR/Cas9. The obtained PCR products were subsequently subjected to sequencing. Within this group, 11 transgenic lines carrying albino mutations represented 61.11% (Table 1). Concurrent mutations of both genes in transgenic plants were referred to as double mutations, while single mutations were the result of mutations in only one gene. Within the

total number of transgenic plants, double mutants constituted 11.11%, while eight *BocPDS1* single mutants accounted for 44.44%, and one *BocPDS2* single mutant accounted for 5.55% (Table 1). In general, the representation of single mutant-type mutations exceeded that of double mutations, and *BocPDS1* exhibited a greater frequency of mutations compared to *BocPDS2*. In the present work, the results indicate that the CRISPR/Cas9 system-induced gene editing when employing endogenous tRNA-processing-system-based approaches was highly successful. Additionally, the sgRNA-expressing cassette may edit both copies of the gene simultaneously.

Table 1. Analysis of mutation efficiency of *BocPDSs* genes in Chinese kale.

Mutation Gene	Number of Mutants	Mutation Rate (%)	Total Number of Mutants	Total Number of Strains Detected	Total Mutation Rate (%)
BocPDS1 + *BocPDS2*	2	11.11			
BocPDS1	8	44.44	11	18	61.11
BocPDS2	1	5.55			

3.5. Analysis of BocPDSs Mutation Patterns

The effects of the *BocPDS1* mutation were further analyzed. A total of 10 *BocPDS1* mutations were identified (Figure 4A), including one that showed a G to A substitution at the twenty-first base upstream of the PAM locus, resulting in an amino acid substitution. Among the remaining nine mutants, an AT or 10 bp (ATTAATATAT) insertion was detected in the third intron of *BocPDS1*, while two of these nine mutants showed a 13 bp (CATACAAAGTTTG) deletion in the fourth intron of *BocPDS1*.

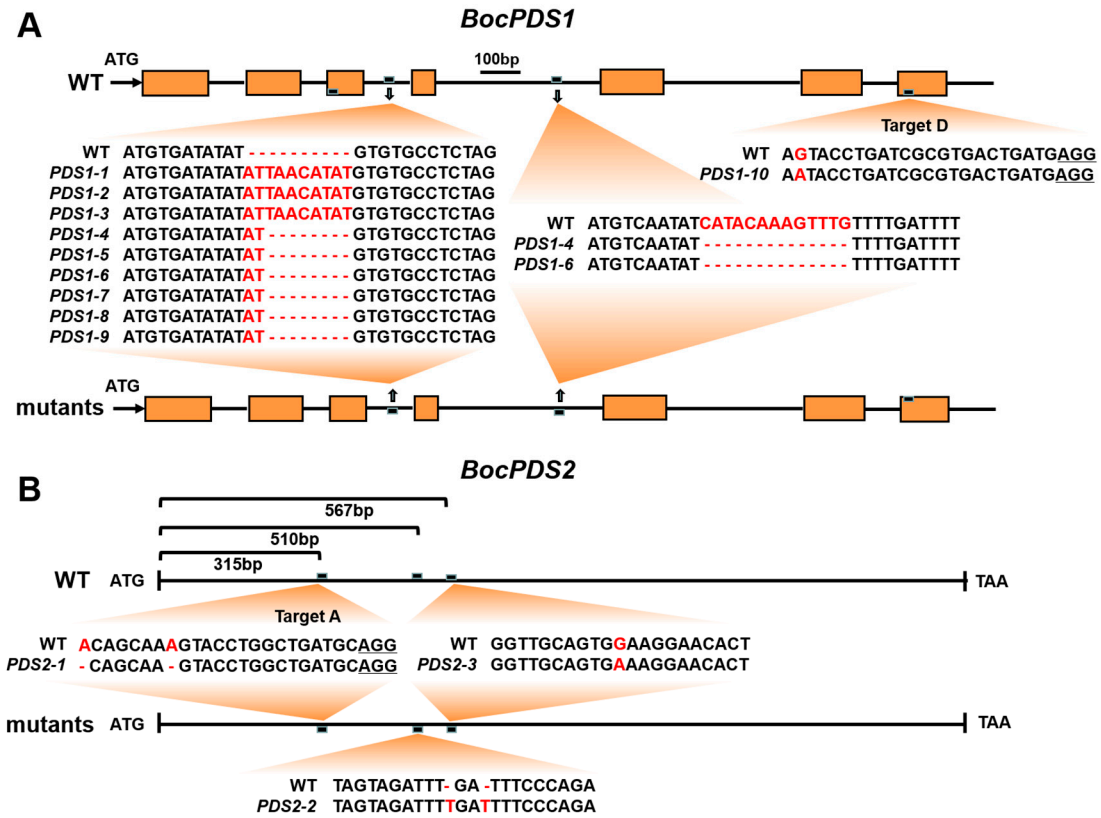

Figure 4. Analysis of mutation type of *BocPDSs* genes in Chinese kale. (**A**) *BocPDS1* mutation types. (**B**) *BocPDS2* mutation types. The PAM sequence (NGG) is highlighted, while altered bases are denoted in red text. The short line denotes the base for deletion.

Similarly, the effects of the *BocPDS2* mutations were also investigated. Unlike PDS1, we found that *BocPDS2* mutations mainly occur in exons or targets. A total of three *BocPDS2* mutations were identified (Figure 4B). Among them, the *BocPDS2-1* mutant showed a deletion of base A at the sixteenth and twenty-third bases upstream of the PAM locus of target site A. This deletion results in the appearance of a stop codon (TGA) upstream of the PAM locus, resulting in a non-functional *BocPDS2* protein. The *BocPDS2-2* mutant showed a substitution of base G to base A at 510 bp downstream of the promoter (ATG). This substitution causes the amino acid to change from tryptophan (Trp) to stop codon (TGA), thus producing a nonsense *BocPDS2* protein. The *BocPDS2-3* mutant showed an insertion of base T at 567 bp downstream of the promoter (ATG). This insertion leads to the appearance of a stop codon (TGA), thereby also generating a truncated (nonsense) *BocPDS2* protein.

This investigation found many mutation types, including base deletions, base substitutions, base insertions, and combinatorial mutagenesis (simultaneous insertions and deletions) (Figure 4). Of these alterations, 61.54% were base insertions, followed by base substitutions at 15.38% and combinatorial mutagenesis. Base deletion mutations constituted merely 7.69% of the documented genomic changes (Table 2). Furthermore, we observed that mutations in targets or exons predominantly consist of single-base deletions, substitutions, and insertions, whereas intron mutations are primarily characterized by AT or ATTAATATAT insertions (Figure 4).

Table 2. Frequency analysis of mutation type of *BocPDSs* genes in Chinese kale.

Mutation Type	Number of PDS1 Mutants	Number of PDS2 Mutants	Total Mutation Rate (%)
Base deletion	0	1	7.69
Base insertion	7	1	61.54
Base substitution	1	1	15.38
Combinatorial mutagenesis	2	0	15.38

3.6. Analysis of Potential Off-Targets

To determine if the CRISPR/Cas9 system, which relies on endogenous tRNA processing, selectively modifies the *BocPDSs* genes, we identified four possible off-target sequences using the CRISPR-GE online portal. Out of the four possible off-target sites, site A demonstrated a 4-base pair mismatch in the starter sequence, site B had a 2-base pair mismatch, site C exhibited a perfect match, and site D only displayed a 1-base pair mismatch (Figure 5). The PCR amplification of all four possible off-target locations was performed using particular primers (Table 2). The sequencing analysis did not identify any mutations in any of the examined locations. The results demonstrate that CRISPR/Cas9-induced target mutagenesis exhibits a high degree of specificity towards the *BocPDSs* in Chinese kale without any unintended consequences.

Figure 5. Potential off-target analysis. The upper row is the target sequence, and the lower row is the potential off-target sequence. The seed sequence is underlined. PAM is highlighted in green. red indicates mutated bases.

4. Discussion

Compared with the ZFNs and TALENs systems, the CRISPR/Cas9 system has an unmatched advantage [32–34]. Both ZFN and TALEN are synthetic, but CRISPR/Cas originates from bacterial sources. In nature, CRISPR/Cas9 is activated when a virus or foreign pathogen infiltrates a bacterium. Utilizing the relevant Cas proteins, the system

seizes and cleaves a segment of the viral DNA, subsequently integrating it into the CRISPR locus of the bacterial genome [35]. Theoretically, an editable location (PAM sequence) can be located in every eight bases of the genome, allowing for the potential editing of nearly all genes; however, gene targeting efficacy varies depending on the species or cell type. Previous results indicate that Fast-cycling *B. oleracea* DH1012 has merely 10% editing efficiency in the *GA4* gene [36] and the editing efficiency of the *PDS* gene and the *SRK* gene in cabbage are 37.5% and 53.5%, respectively [27], which are far lower than the mutation efficiency of 91.6% in rice [37], 89% in Arabidopsis [37], and 87.5% in tobacco [38]. Researchers have continuously optimized and modified the CRISPR/Cas9 system for improved utilization in different species. For instance, a set of efficient gene-editing technologies suitable for cabbage was optimized and obtained [27]. Extending the sgRNA backbone sequence by 5 bp and replacing the fourth base of a string of thymine in sgRNA with cytosine can effectively improve the editing efficiency of CRISPR/Cas9 technology on cabbage and tobacco genes; combining tRNA and HsgRNA to form a polycistronic gene (PTG), which connects two or more sgRNAs in tandem on the same expression vector, to edit multiple sites in the same gene of cabbage and tobacco efficiently [27]. The optimized CRISPR/Cas9 system has the advantages of simpler vector construction and higher targeting efficiency.

Chinese kale (*Brassica oleracea* L.var.) is an important vegetable crop in China. It has a long history of cultivation and high nutritional value. In particular, it is rich in glucosinolate, which has anticancer and anti-aging characteristics and has aroused significant attention [39]. Chinese kale as a diploid is suitable for genetic modification and offspring genetic research. However, compared with rice, tobacco, and other model plants, the genetic transformation efficiency of Chinese kale is lower and the cycle is longer, which brings great difficulties to the research of the gene function of Chinese kale and the application of molecular technologies. The inquiry successfully accomplished fixed-point editing of the Chinese kale genome using the CRISPR/Cas9 system through stable transformation for the first time, thereby enhancing the application of the CRISPR/Cas9 technology in cultivated *Brassica* crops [40]. Chinese kale is a variant of cabbage and has high homology with cabbage, and the optimized CRISPR/Cas9 system has high and precise targeting efficiency in cabbage [27]. Therefore, this study uses the optimized CRISPR/Cas9 system to explore the genome modification efficiency in Chinese kale and achieves a mutation rate of up to 61.11% (Table 1), which was higher than the 37.5% editing efficiency of the *PDS* gene in cabbage [27] and much higher than the 10% editing efficiency of the *GA4* gene in the double haploid DH1012 of the hybrid offspring of Chinese kale and broccoli [36]. This showed that the CRISPR/Cas9 gene editing system based on endogenous tRNA processing can induce high-efficiency mutagenesis in Chinese kale.

The type of mutation is affected by different factors and varies with plant species. It is reported that the type of gene mutation in rice is mainly 1 bp insertion [41], and the type of gene mutation in Arabidopsis is mainly 1 bp deletion [42]. The main types of gene mutations in tobacco are the deletion or replacement of a few bases [43]. The main types of gene mutations in cabbage are deletions of longer fragments and single-base insertions [27]. Gene mutation types in Chinese kale are mainly short nucleotide substitutions [40]. In this study, the types of gene mutations in Chinese kale were mainly short nucleotide insertions. This is different from the research reported by [40]. These differences may be caused by differences in plant genotypes, transformation methods, target genes, or target sites.

This work revealed that two mutations in *BocPDS2* were not located at the target site but rather among the flanking regions or exons. In both of these mutations, a stop codon (TGA) is introduced, leading to the production of a shortened (nonsense) *BocPDS2* protein. On the other hand, we discovered that an AT or 10 bp (ATTAATATAT) insertion was discovered in the third intron of *BocPDS1* in nine mutants. This mutation involves an AT-rich third intron, suggesting that Cas9 may preferentially edit AT-rich regions in Chinese kale, similar to [36]. Nevertheless, this idea demands additional verification. Indeed, a multitude of studies have demonstrated a strong correlation between intronic sequences and the expression of plant

functional genes. Many microRNAs distributed within introns aid their target genes by facilitating both synergistic and antagonistic regulatory effects [44–46]. For example, the mapping of miR838 on the intron of the DCL1 gene has been shown to facilitate gene expression by auto-regulation in *A. thaliana* [43]. In this investigation, we identified mutations in the third and fourth introns of BocPDS1, which are highly prevalent (Figure 4A). Moreover, all of the mutations occurring in the intron gave rise to mosaic albino phenotypes (Figure 3), suggesting a reduction in the activity of the *BocPDS1* gene. Several introns might participate in the regulation of gene expression in Chinese kale. Such a finding aligns with the conclusion drawn in reference [36]. However, the underlying causes of gene function loss after intron mutations are still not fully understood and will be intriguing for future research. Interestingly, *BocPDS1* and *BocPDS2* double mutations were pure albino phenotypes, and *BocPDS1* or *BocPDS2* single mutations resulted in a mosaic albino phenotype, which indicates that the functions of *BocPDS1* and *BocPDS2* have overlapping but complementary mechanisms (Figure 3). In addition, the loss of function of either gene resulted in albino phenotypes, suggesting that both are essential for chlorophyll biosynthesis.

The frequency of off-target mutations induced by CRISPR/Cas9 is typically well below that caused by chemical and physical mutagenesis [1]. Many groups reported that they did not identify any mutations in potential off-target sites [2–4]. However, the CRISPR/Cas9 gene editing technology has been reported to have off-target events in many studies [5,6]. The main factor that determines the specificity of sgRNA is the 10–12 bp seed sequence of the PAM region. If the potential off-target site has only 4 bases or fewer that are not matched with the target site, and the rest of the bases are completely complementary paired, the site may be mutated to produce off-target effects [7,8]. This work evaluated potential off-target regions and found none, demonstrating that CRISPR/Cas9 in Chinese kale exhibits great specificity. Nevertheless, we identified just four locations with a significant probability of off-target effects and did not comprehensively assess them using whole genome sequencing; more potential off-target sites require future analysis. Significantly, there is increasing evidence that off-target effects may not pose a substantial issue in plants, as their real risk during tissue culture-based transformation appears to be minimal [9]. In addition, any off-target modification in plants can be removed by segregation. During the mutation detection process, it was found that the four target sites were in full compliance with the sgRNA design rules. However, two of the target sites did not undergo editing, and no off-target effects were detected. Other researchers have also found similar problems, which may be due to the complex chromosome structure or other reasons, but the specific reasons are not yet clear and need to be further studied [47]. In addition, the CRISPR/Cas9 gene editing system established using *PDS* genes can also be stably and efficiently applied to other important functional genes, and the induced mutations can be stable in inheritance, thus contributing to crop improvement [8]. Therefore, establishing a stable and efficient gene editing system is a very critical step. In this study, the establishment of a stable and efficient gene editing system for Chinese kale and other brassica crops provides the theoretical basis and technical support for further functional verification of key genes and crop quality improvement.

5. Conclusions

The rapid advancement and ongoing refinement of the CRISPR/Cas9 system have rendered it a potent instrument for plant genome study, and its application is expanding to include an increasing number of species. The present work integrated the CRISPR/Cas9 system with endogenous tRNA processing to accomplish targeted modification of the Chinese kale genome by stable genetic transformation. The results demonstrate that the CRISPR/Cas9 gene editing system, which relies on endogenous tRNA processing, can effectively induce mutagenesis in Chinese kale. This study establishes a theoretical basis and technical assistance for the more efficient implementation of CRISPR/Cas9 gene-editing technology in Chinese kale.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10121244/s1, Table S1: Primers used in the study.

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