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RESEARCH ARTICLE

Targeted Editing of the *StPDS* Gene using the CRISPR/Cas9 system in Tetraploid Potato

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ABSTRACT

The CRISPR/Cas9 system has been applied in a variety of plants for targeted gene editing due to its accuracy, high efficiency, and low price. It has been reported that the CRISPR/Cas9 system can lead to mutation in potato, however, the mutation patterns and genotypes of CRISPR/Cas9-induced mutants in tetraploid potato plants have not yet been disclosed in detail. In the present study, the *Solanum tuberosum* phytoene desaturase (*StPDS*) gene was selected for inducing targeted mutagenesis. The obtained *StPDS* knockout mutants exhibited a mutation frequency of 46.67% and clear albino phenotypes. All transgenic plants were tested for the hygromycin resistance gene and a high positive transformation frequency (96.77%) was observed in all tested resistant plants. Genotype analysis of the mutants revealed that heterozygotes accounted for 35.51%, chimeras accounted for 64.29%, and no homozygotes and biallelic mutations were detected. The patterns of the detected mutations consisted mainly of 1 to 2-nucleotide insertions and deletions, followed by 2-bp replacements. In short, the results of this study prove that the CRISPR/Cas9 system is an effective approach for cultivated potato gene engineering.

Keywords: Gene editing; CRISPR/Cas9; Tetraploid potato; *StPDS*

INTRODUCTION

Potatoes are one of the five staple foods in China. It is the third most important food crop in the world with high nutritional value, strong adaptability, and high yield. Potato is clonally propagated plant so that it is complicated and time-consuming to breed new cultivars via traditional cross-breeding methods. In addition, the tetrasomic inheritance of the cultivated potato (*Solanum tuberosum*) makes it a challenge to conduct the breeding and research of potato using traditional cross-breeding methods (Muthoni et al., 2015). Fortunately, with the development of molecular biology, these challenges can be solved by genetic modifications that allow the dominant down-regulation of endogenous genes. However, although down-regulation approaches are efficient, problems may emerge in the long run since the endogenous genes are still expressed, resulting in the potential instability of the desired trait after several generations (Rajeevkumar et al., 2015). In recent years, the development of gene editing approaches has allowed for the targeted mutagenesis of polyploid species, providing an efficient strategy to cultivate new crop varieties.

Genomic site-directed editing uses sequence-specific nucleases (SSNs) to cleave DNA double strands at the target site of the genome, resulting in DNA double-strand breaks (DSBs) that will be repaired through homologous recombination repair (HR) or non-homologous end joining (NHEJ) pathways, resulting in nucleotide insertion, deletion, or DNA fragment replacement in the targeted gene (Andersson et al., 2017; Jinek et al., 2012). Currently, SSNs have been applied to crop genetic improvement through Zinc finger nucleases (ZFNs) (Ainley et al., 2013; Bibikova et al., 2002; Dreier et al., 2005), transcription activator-like effector nucleases (TALENs) (Christian et al., 2010; Li et al., 2011), and clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas system) (Cong et al., 2013; Mali et al., 2013; Shalem et al., 2015). The CRISPR/Cas9 is the newest technology and its application prospect is the most extensive among these three SSNs.

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To date, the CRISPR/Cas9 system has been used to genome targeted mutagenesis in various plants, including *Arabidopsis thaliana* (Feng et al., 2014), rice (Zhang et al., 2014), *Nicotiana benthamiana* (Nekrasov et al., 2013), wheat (Wang et al., 2014), corn (Liang et al., 2014), sorghum (Jiang et al., 2013), tomato (Xu et al., 2016), and potato (Andersson et al., 2017; Butler et al., 2015; Wang et al., 2015). Although the CRISPR/Cas9 system was employed in potato to generate mutants, the patterns and genotypes of CRISPR/Cas9-induced mutation in the potato T0 generation still require careful analysis.

The phytoene desaturase gene (*PDS*) encodes a significant enzyme involved in carotenoid biosynthesis. In other plant species, *PDS* mutants plants show a clearly albino phenotype and have served as a model for CRISPR/Cas9-mediated target gene editing (Fan et al., 2015; Nekrasov et al., 2013; Shan et al., 2013). In the present study, we utilized the *Solanum tuberosum* PDS (*StPDS*) as the target gene in an *Agrobacterium*-mediated genetic transformation. Our study investigates the mutation patterns and genotypes of CRISPR/Cas9-induced mutants in tetraploid potato (Xuanshu 2). The findings presented here will lay a basis for the CRISPR/Cas9 targeted editing technology in cultivated potato.

MATERIALS AND METHODS

Plant materials

The tetraploid potato Xuan Shu 2, which is one of the main cultivars in Southwest China, was used in this study; the virus-free seedlings were provided by the Bijie Institute of Agricultural Science. The stem segments of two to three-week-old virus-free seedlings were used for *Agrobacterium* transformation and were grown in MS medium under the conditions of on 16-h light/8-h dark at 23 °C.

Vector construction

The CRISPR/Cas9 vectors consisted by two basic vectors pSG and pCC were constructed as previously described (Sun et al., 2018). The gene sequences of *PDS* of potato (gene ID: PGSC0003DMT400023666) were downloaded and obtained from The Spud DB database (http://solanaceae.plantbiology.msu.edu). The *StPDS* Sequence was analyzed through the online tool of CRISPR-P (http://crispr.hzau.edu.cn) and the target sequence of *StPDS* was designed. The gene fragment flanking regions of the selected target site of *StPDS* in Xuanshu 2 was cloned and aligned to verify the consistency of the target sequence. The single guide RNA (sgRNA) expression cassette was digested from the pSG-*StPDS* recombinant plasmid, and purified by gel recovery, and then inserted into the pCC vector to generate pCC-target-sgRNA for potato transformation.

Agrobacterium-mediated transformation of potato

Agrobacterium-mediated transformations of potato were performed as follows. In brief, the stem segment of potato virus-free seedlings grown for 2-3 weeks was pre-cultured for three days in MS medium with 0.5 mg L^{-1} 2,4-D, 1.0 mg L^{-1} 6-BA, and 0.8% phytagar and was then infected with the overnight cultured *Agrobacterium* strain GV3101 for 1-2 min. The infected explants were co-cultivated in MS medium for three days, and transferred to the MS medium with 0.5 mg L^{-1} GA_3, 2 mg L^{-1} BA, 2 mg L^{-1} ZT, 0.8% phytagar, 325 mg L^{-1} carbenicillin, and 325 mg L^{-1} timentin for seven days. Hygromycin-resistant shoots were regenerated on the same medium with 8 mg L^{-1} hygromycin B, and transferred to the subculture medium. The hygromycin-resistant seedlings were gained after 2-3 months, and used for following analysis.

Transformation efficiency detection

Genomic DNA of the hygromycin-resistant and wild-type (WT) plants was extracted according to a standard cetyltrimethylammonium bromide (CTAB) method. To confirm the transformants of the potato cultivar, polymerase chain reaction (PCR) amplification were conducted with the specific primers HygR-F and HygR-R (Table 1). The transformation efficiency was calculated according to the results of electrophoresis of the PCR products. Only the transformants with the hygromycin gene were selected for later analysis.

Mutation detection

Genomic DNA of each positively transgenic plant was amplified using *TransStart FastPfu* Fly DNA Polymerase (TransGene, Beijing) and specific primers (*StPDS*-CRISPR test-F and *StPDS*-CRISPR test-R, Table 1) which were designed to amplify the 555-bp flanking regions of the target site of *StPDS*. The PCR products were purified and cloned into the *pEASY*-Blunt vector (TransGene, Beijing); the resulting plasmids were used to transform *E. coli* strain JM109 (TaKaRa, Japan). About 9-11 white colonies were selected from each plate that contained the DNA of the positively transgenic plants for sequencing. DNAMAN software was used to compare the sequences, and the mutation rate was calculated, as well as the mutation type and genotype were analyzed based on all the sequencing data (Sun et al., 2018).

Color determination

The color of the 14 mutants was measured with an NR110 chromameter (3nh, Shenzhen). Three positions of each mutant were randomly selected and the color was recorded as L^*, a^*, and b^*. The + L^* direction is white, - L^* direction is black, + a^* direction is red, - a^* direction is green, + b^* direction is yellow, and the - b^* direction is blue. The a^* or b^* value is biased towards 0, which represents white.

Table 1: The primers of *StPDS* mutants

Primer names	Sequence of primers (5'-3')	Annealing temperature (°C)	Amplified fragment length (bp)	Aims
sgRNA-*StPDS*-F	TCACAAACCGATACTACTGG	55	23	The synthesis of target site
sgRNA-*StPDS*-R	CCAGTAGTATCGGTTTGTGA			
StPDS-CRISPR test-F	ACAATGCTGGAGCAGTGACCTC	57	555	Detect the mutation of transgenic plants
StPDS-CRISPR test-R	GCATAGAACAAACTAACAGAGAGGGGT			
Hyg-F	CGATTGCGTCGCATCGACC	58	558	Detect the hygromycin resistance gene
Hyg-R	TTCTACAACCGGTCGCGGAG			

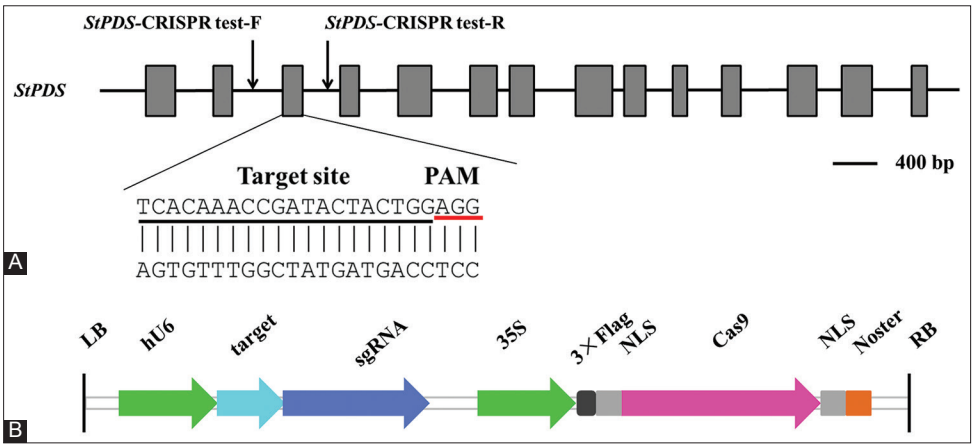

Fig 1. Map of the constructed vector. (A): Schematic of the *StPDS* gene with the sgRNA target site and sequence. ▌ indicates exon. ▬ indicates intron; (B): Structure of the CRISPR/Cas9 binary vectors for potato transformation. The Cas9 cassette is driven by the 35S promoter, while the sgRNA is controlled by the hU6 promoter. NLS, nuclear localization sequence.

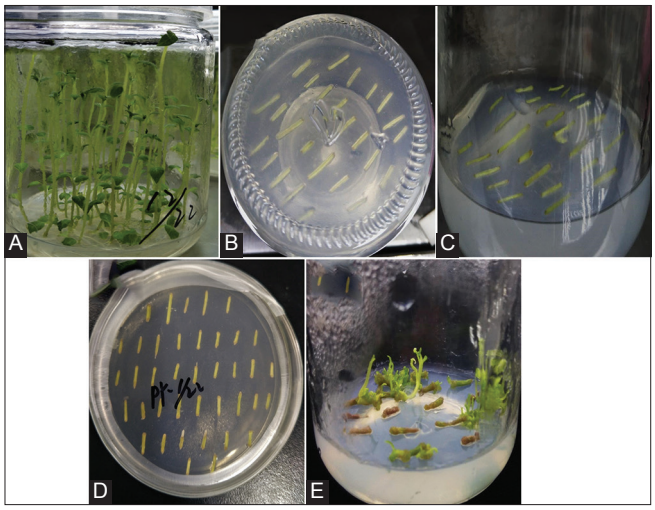

Fig 2. The procedure of *Agrobacterium*-mediated genetic transformation in potato. A: Potato virus-free seedling; B: pre-culture; C: co-culture; D: delayed screening; E: resistance screening.

RESULTS

Assessment of transformation efficiency

The target sequence and protospacer adjacent motif (PAM) are shown in Fig. 1A. The recombinant plasmid of pCC-target-sgRNA was constructed (Fig. 1B), and then about 1800 explants were carried out for Agrobacterium-mediated transformation. Thirty-one hygromycin-resistant plants were gained. The results of PCR amplification showed that a 558-bp target band was observed in the plasmid (positive control), the empty vector, and the 30 resistant plants, while no bands were found with the template in 1 out of the 31 plants (lines 29) and WT plants (negative control) (Fig. 3), indicating that the target expression cassette was successfully transferred into the 30 potato lines. Moreover, the false positive rate of the CRISPR/Cas9 expression vector of the potato plants was only 3.33%.

Analysis of *StPDS* gene mutations

The mutations in *StPDS* mediated by the CRISPR/Cas9 system were analyzed (Fig. 4). Fourteen StPDS mutants were obtained by sequencing confirmation. It was found that most of the mutation lines (M1, M3, M5, M6, M8, M11, M14, M16, M17, M22, M26, M30) were deletion or insertion mutations of 1-2 A bases in the region enriched in A bases downstream of the target site, which led to frameshift mutations that affected gene function. M25 had a 2-bp replacement near the target site, leading to an alteration in its relevant amino acid; M27 had a 2-bp replacement near the target site, leading to an alteration in its relevant amino acid and there was also a single base deletion in the region enriched in A bases downstream from the target site, resulting in frameshift mutation.

Table 2: Efficiency of CRISPR/Ca9 system in causing *StPDS* gene mutations

Target gene	No. of plants examined	No. of plants with mutations	Mutation rate (%)	Bi-allelic		Homozygous		Heterozyous		Chimeric	
				Number	Ratio (%)	Number	Ratio (%)	Number	Ratio (%)	Number	Ratio (%)
StPDS	30	14	46.67	0	0	0	0	5	35.51	9	64.29

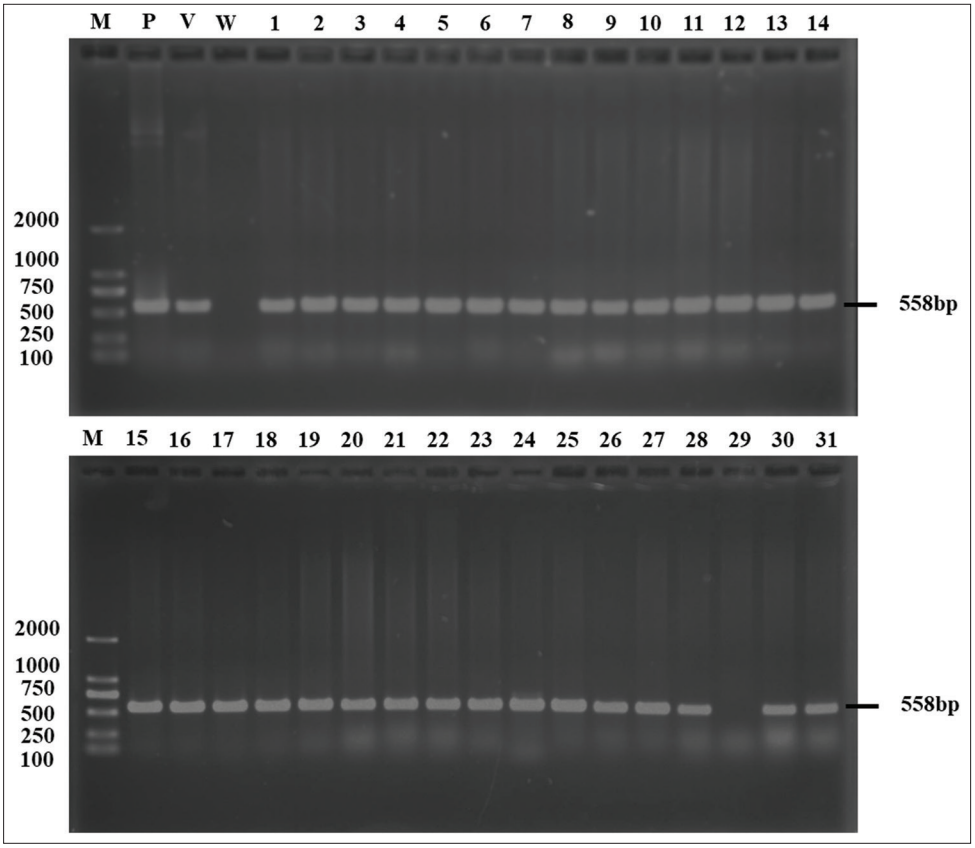

Fig 3. PCR detection of the hygromycin resistance gene for the estimation of transformation efficiency. M: DL2000 maker; P: Positive control plasmid; V: empty vector; N: water as the negative control; W: wild-type which is the non-transgenic plant; 1-31: indicates the resistant plant line number.

We also detected the mutation efficiency of CRISPR/Cas9 in the StPDS. Approximately 46.67% (14/30) of the transgenic plants harbored mutations. Among all of the mutants, five heterozygous mutations were found, accounting for 35.51% of the total mutation rate and nine chimeric mutations were found, accounting for 64.29% of the total mutation rate; no bi-allelic and homozygous mutation occurred (Table 2).

Genotypes of *StPDS* mutants

To investigate the genotypes of StPDS mutants induced by the CRISPR/Cas9 system, all 14 mutants were detected and the data are listed in Table 3. In all heterozygotes, some mutants (M5, M8, M14, M17) had 1-bp deletion and WT mutation; another mutant (M25) had 2-bp replacement and WT mutation. This indicated that the mutations occurred in an embryogenic cell before the cell underwent division. The most abundant genotype was chimera (9/14), whose cells had multiple types of mutations, including nucleotide insertion, deletion, and wild-type. This finding indicated that the mutations occurred after the division of the transformed embryogenic cell.

Patterns and frequency of *StPDS* mutations

Among the mutation types, around 50% of the mutations were insertions, followed by deletions (46%), whereas replacements accounted for only 4% (Fig. 5). All mutations were short nucleotide changes (1-2 *bp*), while 1-bp mutations were the main type (80%); among these, 1-bp deletion mutations accounted for 44% of the observed mutations and 1-bp insertion mutations accounted for 36%.

Phenotype and color of *StPDS* mutants

We observed the phenotype of the StPDS mutation and found that after StPDS was knocked out, the potato mutants exhibited a clear albino phenotype compared to the WT plants and empty vector (EV) transformed plants (Fig. 6), indicating that the function of the StPDS gene

Fig 4. CRISPR/Cas9 system-induced mutation detection in potato mutants. The target sequence is indicated in blue, the PAM sequence (NGG) is underlined in red, the mutated bases are shown in red font, and the red dot represents the deleted bases. i #, # number of bases insertion. d #, # number of bases deletion. r #, # number of bases replacement. The number of single clones with the same mutation is shown in parentheses.

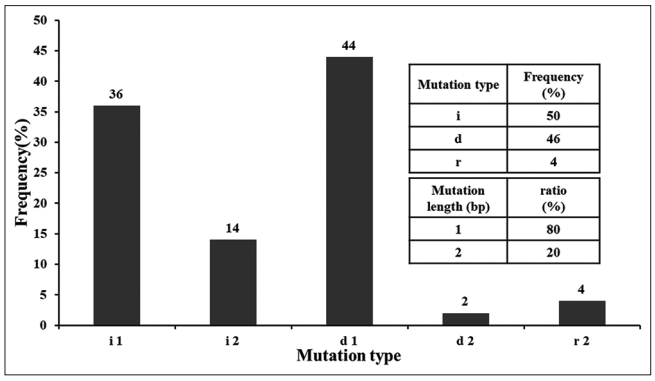

Fig 5. Pattern and frequency of mutations in potato using CRISPR/Cas9-mediated gene editing. The graph shows the sequencing data of all mutants. The upper right illustration shows different types of mutations. The lower right illustration shows mutations of different lengths. X-axis: i #, the number of base insertion. d #, the number of base deletion; r #, the number of base replacement. Y-axis: indicates the frequency of the mutation.

Table 3: The genotypes of the *StPDS* mutants

Target gene	Lines	Zygosity	Genotype
StPDS	M1	Chimera	5i1, 3i2, 1wt
	M3	Chimera	1i1, 1d1, 7wt
	M5	Heterozygote	2d1, 7wt
	M6	Chimera	2d1, 3i1, 5wt
	M8	Heterozygote	3d1, 7wt
	M11	Chimera	3i1, 2i2, 4wt
	M14	Heterozygote	4d1, 6wt
	M16	Chimera	2i2, 3i1, 6wt
	M17	Heterozygote	1d1, 10wt
	M22	Chimera	4d1, 2i1, 5wt
	M25	Heterozygote	1r2, 8wt
	M26	Chimera	1d1, 1i1, 8wt
	M27	Chimera	1r2, 3d1, 6wt
	M30	Chimera	1d1, 1d2, 8wt

*d#, # of bp deleted at target *StPDS*; i#, # of bp inserted at target *StPDS*; r#, # of bp replaced at target *StPDS*; WT, wild-type sequence with no mutation detected. The number before i, r, d, wt represents the number of clones of the mutation.

Table 4: The color values of *StPDS* mutants

Lines	*L**	*a**	*b**
WT	33.03±0.50^{fg}	-1.97±0.32^{i}	-1.07±0.23^{g}
EV	32.89±0.23^{g}	-1.51±0.08^{h}	-1.81±0.52^{h}
M1	37.75±0.26^{a}	0.68±0.09^{cde}	1.37±0.13^{d}
M3	37.36±0.43^{ab}	0.81±0.18^{bcd}	1.47±0.13^{d}
M5	35.09±0.28^{e}	0.08±0.09^{g}	-1.03±0.14^{g}
M6	37.97±0.30^{a}	1.17±0.13^{a}	1.39±0.09^{d}
M8	36.69±0.23^{bc}	0.81±0.12^{bcd}	1.50±0.42^{d}
M11	36.32±0.16^{cd}	0.48±0.17^{ef}	1.66±0.28^{cd}
M14	35.30±0.29^{cd}	0.88±0.09^{bc}	0.80±0.28^{e}
M16	37.42±0.66^{ab}	0.98±0.01^{ab}	2.04±0.13^{bc}
M17	35.56±0.15^{de}	0.20±0.05^{g}	0.79±0.13^{e}
M22	36.81±0.51^{bc}	0.57±0.07^{def}	1.39±0.01^{d}
M25	33.81±0.21^{f}	0.08±0.06^{g}	-0.47±0.14^{f}
M26	36.04±0.48^{cd}	0.57±0.10^{def}	1.36±0.14^{d}
M27	35.67±0.61^{de}	0.80±0.16^{bcd}	2.69±0.17^{a}
M30	36.27±0.62^{cd}	0.30±0.08^{fg}	2.45±0.29^{ab}

was entirely or partially silent. The color analysis results of the StPDS mutants are shown in Table 4. The L* values of all mutants were remarkably higher than those of the WT and EV plants, except for M25. The a* values of all the mutants were remarkably higher than those of the WT and EV plants; the values were positive and the absolute values were close to 0. The b* values of all of the mutants were also significantly higher and were positive, except for M5 and M25 (Table 4).

DISCUSSION

CRISPR/Cas9 is a highly suitable tool for both applied and basic research, and the method has been used to various plants. *Agrobacterium*-mediated genetic transformation is the most common approach for plant transgenic technology (Sheng et al., 2016). Stem sections of test-tube seedlings and potato slices in vitro are the most commonly used explants for potato genetic transformation (Heeres et al., 2002; Chakravarty et al., 2007). However, taking into account the development of virus-free potato cultivation in production, its expansion is based on stem propagation. Therefore, in order to provide technical guidance and convenience for potato researchers for production applications, we selected potato stem segments as *Agrobacterium*-mediated genetic transformation explants. We transferred the CRISPR/Cas9 vector containing the recombinant plasmid pCC-StPDS-sgRNA and obtained 14 strains of *StPDS* gene knockout mutants with a mutation rate of 46.67%. This result is consistent with the mutation rate through CRISPR/Cas9 system in the callus of diploid (X914-10) and tetraploid (Désirée) potatoes (Butler et al., 2015). The mutants exhibited an albino phenotype, which is consistent with the research results of Tian et al. (2017), who conducted targeted editing of the *ClPDS* gene in watermelons by CRISPR/Cas9 (Tian et al., 2017).

It is reported that homozygous and biallelic allele mutations were found in T0 mutants induced by CRISPR system mutations. Zhang et al. edited of 11 target genes in two rice subspecies via the CRISPR/Cas9 system; a significant percentage of the edited genes of the T0 lines were homozygous or biallelic (Zhang et al., 2014); Gao et al. used the CRISPR/Cas9 system for targeted editing of the *NtPDS* and *NtPDR6* genes in *Nicotiana tabacum* with biallelic mutation rates of 36.4% and 6.26%, respectively (Gao et al., 2015). In this study, although our data suggested the effectiveness of the CRISPR/Cas9 system in potato, no biallelic and homozygous mutations were found in any of the mutants. There are multiple reasons for this observation. First and foremost, the genetic background of tetraploids is complex and their gene editing is rather difficult. In addition, DSBs and mutations may be slow

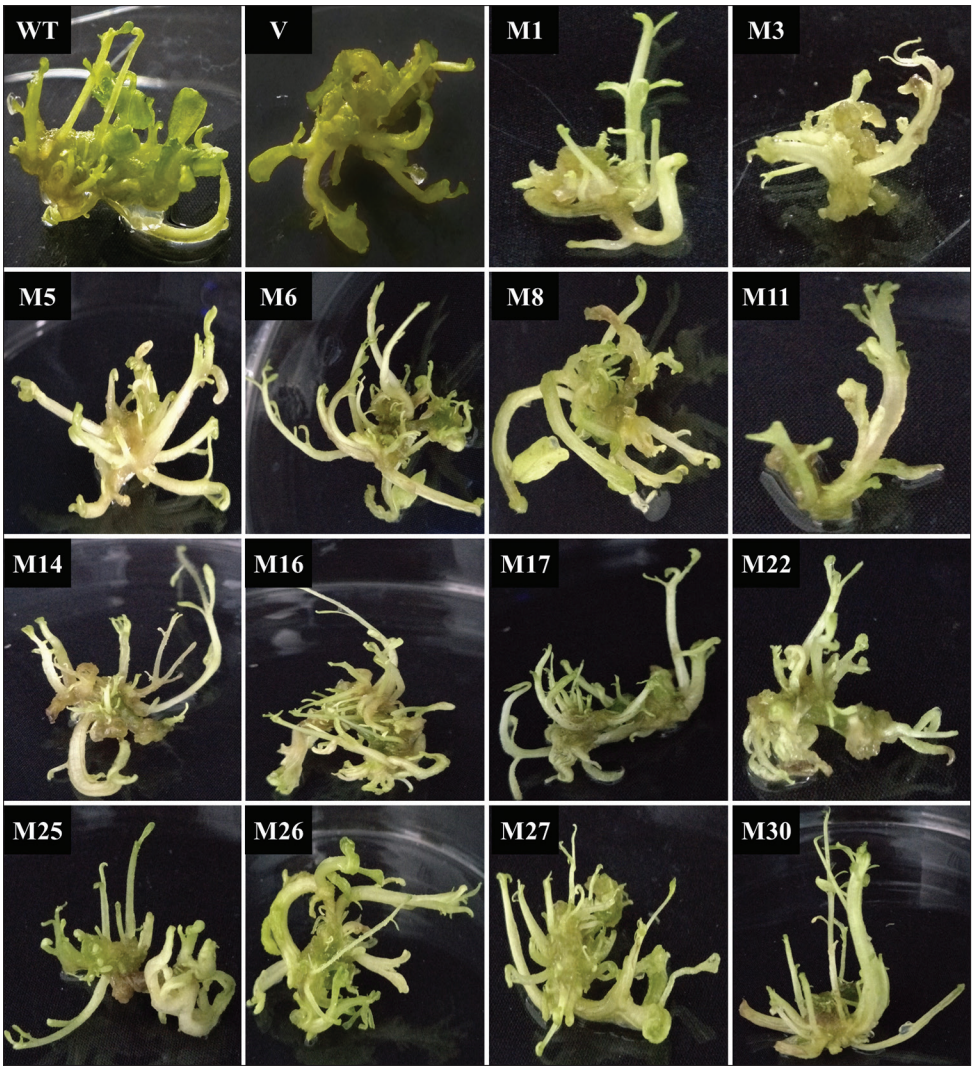

Fig 6. The albino phenotype of the *StPDS* mutants after transformation with the CRISPR/Cas9 vector. WT: wild type; EV: the transgenic plants with the empty vector (contain T-DNA); M#: indicates the mutant number; Scale bar 5 mm.

when the activity of the CRISPR/Cas9 system is low. Lastly, there is a possibility that a high proportion of faultless repairs did occur after the DSB. Furthermore, no homozygous mutations were detected; this might because of the significance of the target gene regarding plant growth and development (Zhang et al., 2014).

In this study, insertions, deletions, and replacements were all found in the detected clones. All insertion mutations were 1-2 bp insertions of A nucleotides; this result is in agreement with the previous studies (Pan et al., 2016; Zhang et al., 2014). All deletion mutations were short nucleotide changes, similar to previous studies of gene editing in potato using CRISPR technology (Andersson et al., 2017; Butler et al., 2015; Morineau et al., 2017; Wang et al., 2015). Lawrenson et al. (2015) reported that large deletion mutations were observed at the *BolC.GA4.a.* target site in *Brassica oleracea*. However, in our study, there were no large deletion mutations and this may be attributed to the discrepancies in DNA-repair mechanisms between different species.

This study had several limitations. First, most of the mutations were found at the flanking regions of target site and a similar result was observed in our previous research (Sun et al., 2018). In this study, with the exception of strain 25, the other strains mutated downstream of the target sequence at the location of the A nucleotide enrichment (Fig. 4). There are multiple reasons for this observation. First, the use of the CRISPR/Cas9 vector that contains an hCas9 protein may have caused lower editing efficiency; in addition, different crop species such as potato and rice often result in different efficiencies (Butler et al., 2015; Xie et al., 2013). Second, a relatively low gene editing efficiency (only 46.67%) was obtained in this study. This may be attributed to the low cleavage activity of hCas9. Additionally, there may be a better approach to repair DNA in potato. There is evidence that the optimization of the

codon can promote the expression level of the Cas9 gene (Li et al., 2013; Johnson et al., 2015). In a follow-up study, we will perform codon optimization of Cas9 to improve the accuracy and efficiency of genetic editing in potato. In summary, although our CRISPR/Cas9 vector was not perfect, the results of the phenotype and sequencing analysis of the mutants indicate that the CRISPR system effectively performs targeted editing on the tetraploid potato cultivar Xuanshu 2.

CONCLUSIONS

In the present study, the CRISPR/Ca9 system was used for targeted editing of the *StPDS* gene in potato and *StPDS* gene knockout mutants were obtained through *Agrobacterium*-mediated genetic transformation. The total mutation rate was 46.67%; the heterozygotes accounted for 35.51% and the chimeras accounted for 64.29%. The mutations were dominated by a short nucleotide (1-2 bp) change. All of the mutants exhibited a clear albino phenotype. The results highlight the successful use of the CRISPR system for the tetraploid potato cultivar Xuan Shu 2 and lay the foundation for molecular breeding and gene function research of cultivated potatoes.

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Authors' Contribution

Bo Sun, and Fen Zhang conceived and designed the experiments; Jie Ma, Ai-Hong Zheng, and Qiao Yuan performed the experiments; Ping Zhou, Rui Wu, Chun-Yan Chen, and Xian-Zhi Wu analyzed the data; Jie Ma, Ai-Hong Zheng, and Ping Zhou Wrote the paper.

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