Journal of Genetic Engineering and Biotechnology 22 (2024) 100380

Contents lists available at ScienceDirect

Journal of Genetic Engineering and Biotechnology

journal homepage: www.elsevier.com/locate/jgeb

CRISPR/Cas9 based genome editing of *Phytoene desaturase* (PDS) gene in chilli pepper (*Capsicum annuum* L.)

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ARTICLE INFO

Keywords:
Biolistic delivery
Chilli pepper
CRISPR/Cas9
Phytoene desaturase
T7E1 assay

ABSTRACT

An effective CRISPR/Cas9 reagent delivery system has been developed in a commercially significant crop, the chilli pepper using a construct harboring two distinct gRNAs targeting exons 14 and 15 of the *Phytoene desaturase* (*CaPDS*) gene, whose loss-of-function mutation causes a photo-bleaching phenotype and impairs the biosynthesis of carotenoids. The construct carrying two sgRNAs was observed to create visible albino phenotypes in cotyledons regenerating on a medium containing 80 mg/L kanamycin, and plants regenerated therefrom after biolistic-mediated transfer of CRISPR/Cas9 reagents into chilli pepper cells. Analysis of CRISPR/Cas9 genome-editing events, including kanamycin screening of mutants and assessing homozygosity using the T7 endonuclease assay (T7E1), revealed 62.5 % of transformed plants exhibited successful editing at the target region and displayed both albino and mosaic phenotypes. Interestingly, the sequence analysis showed that insertions and substitutions were present in all the plant lines in the targeted *CaPDS* region. The detected mutations were mostly 12- to 24-bp deletions that disrupted the exon–intron junction, along with base substitutions and the insertion of 1-bp at the protospacer adjacent motif (PAM) region of the target site. The reduction in essential photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoid) in knockout chilli pepper lines provided further evidence that the *CaPDS* gene had been functionally disrupted. In this present study, we report that the biolistic delivery of CRISPR/Cas9 reagents into chilli peppers is very effective and produces multiple mutation events in a short span of time.

1. Introduction

Among all solanaceous crops, the chilli pepper (*Capsicum annuum* L.) has been reported as most resistant to *in vitro* morphogenesis and plant transformation.[1] It is also a widely distributed, commercially important horticultural crop of *Solanaceae*. This spice crop originated in Mexico and has spread across the globe and is widely cultivated throughout the world as a source of vitamins and minerals that ameliorate nutritional imbalances.[2,3] Due to its high quantities of carotenoids, chlorophylls, ascorbic acid, vitamins, and flavonoids, pepper fruit is increasingly attracting interest as a nutraceutical product in addition to its agricultural significance.[4–6] Additionally, several pepper cultivars contain a group of particular alkaloids called capsaicinoids, which are responsible for their distinctive pungent and 'hot' sensation, which exhibit anti-cancer properties.[7] They have also been shown to have other beneficial effects on human health[8] including alleviation of cough, rheumatism, sore throat, and toothache.[9] Moreover, the phenolic compounds of chilli pepper have high potential as effective antioxidants that may safeguard the immune system against reactive oxygen species (ROS), thereby preventing cancer, cardiovascular illnesses, and neurological diseases.[10,11] In addition to their anti-aging and depigmenting abilities, these metabolites have anti-inflammatory and antibacterial activities also[12] demonstrating a significant potential for their nutritional enhancement via genetic improvement.[8]

The advancement of genome editing technologies such as the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-Associated Nuclease 9 (Cas9) system, which was inspired by studies on the prokaryotic adaptive immune system has enabled precise modification of plant genomes as a biosafe alternative to genetic transformation technology to improve desirable agronomic characteristics in crop plants.[13] In this technology, a single guide RNA (sgRNA) recognizes and directs the Cas9 nuclease to cleave the appropriate target sequence. The resultant double-strand breaks (DSBs) produced in

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https://doi.org/10.1016/j.jgeb.2024.100380
Received 10 February 2024; Received in revised form 8 April 2024; Accepted 21 April 2024

the genomes of targeted crops were repaired via non-homologous end joining (NHEJ) or homology-directed repair (HDR) processes.[14,15] In crops, the NHEJ system stands as the predominant mechanism demonstrating high efficiency in generating gene knockouts by introducing random insertions or deletions (InDels) of nucleotides into the targeted gene.[15] HDR requires a DNA repair template whose sequence is homologous to the sequence flanking the genomic DSB region resulting in precise DSB repair.[13,16]

In the recent past, the utilization of CRISPR/Cas9-based genome editing has attained a rising trend in agricultural biotechnology. This technology is being more frequently employed to improve agricultural productivity, nutritional value, and resilience to both biotic and abiotic stresses in various crops.[17] Although the CRISPR/Cas9 technology demonstrates high efficiency in precise genome editing, the delivery of genome editing components into plant cells is still challenging due to polyploidy, genomic rearrangements, and rigid cell walls. Therefore, a comprehensive analysis of the introduction of CRISPR components to plant cells is vital to achieving effective genome editing.[18]

Several recent studies have demonstrated the effective genome editing in different solanaceous crops including tomato, potato, and brinjal.[19,20] However, the use of such genome editing and integrated approaches in chilli pepper needs to be focused.[19,21] Owing to technical constraints, including the nonresponsiveness to existing tissue culture protocols used for recovering the genome edited lines, this species has remained recalcitrant to genetic transformation.[19] Several attempts have been made to overcome the existing bottlenecks associated with transformation and recovery of chili peppers. For example, transformation efficiency was enhanced when *Brassica napus* BABY BOOM AP2/ERF transcription factor was expressed in pepper cells.[1]

The *PDS* gene encoding the *phytoene desaturase* enzyme, a key enzyme involved in carotenoid biosynthesis pathway.[22] It has been targeted in many CRISPR/Cas mediated genome editing studies as a visible marker to standardize the delivery of CRISPR reagents into plant cells.[23–25] By inhibiting chlorophyll, carotenoid, and gibberellin production, the disruption of PDS results in photobleaching, albino phenotype, and dwarfism.[26] It also allows for simple screening of mutant lines.[27,28] Recent studies have shown the establishment of efficient genome editing using the *PDS* gene through the CRISPR/Cas9 system in several crops, including potato,[24] pea,[29] papaya,[30] coffee,[31] chickpea,[32] hop,[23] onion[25] and squash.[33] Nevertheless, the development of a biolistic-mediated CRISPR/Cas9-reagent delivery system using the *PDS* gene in chilli pepper has not yet been reported. The present investigation concentrates on the development of this system in chilli peppers.

2. Materials and methods

2.1. Plant material for explants preparation

The 10-d old *invitro* raised cotyledons of chilli pepper (cv G4) were used as explants. Sterilized seeds were germinated on a 0.5X MS basal medium (MSB) [34] supplemented with MSB5 vitamins and 2 % (w/v) sucrose. The pH of the medium was adjusted to 5.8 and it was solidified with 0.8 % w/v agar (Himedia, India). The growth conditions included a 16-hour photoperiod provided by 110 W fluorescent lamps (Phillips, India) with a photosynthetic photon flux (PPF) of 65 μmol m^{-2} s^{-1} and maintained at a temperature of 25 ± 1 °C, which was in accordance with our established protocol.[35]

2.2. In silico identification of PDS gene in the Capsicum genome

The Sol Genomics Network (https://solgenomics.net, accessed on 1st December 2021) was utilized in a BLAST search against the reference Capsicum genome for obtaining putative *CaPDS* coding sequence homologous to the completely-annotated *PDS* gene sequences of tobacco, tomato, soybean, banana, and maize were used as a query sequence to obtain the putative *CaPDS* gene sequence (Fig. S1). Then, the functional domains of the *CaPDS* were analyzed and annotated using the PROSITE database (accessed on January 5, 2022, at https://prosite.expasy.org). Following this, the *CaPDS* coding sequence was aligned alongside five other plant *PDS* coding sequences accessible in the NCBI database using the NGPhylogeny online tool (https://ngphylogeny.fr, accessed on 21st May 2021) and employing the MAFFT Alignment method.

2.3. gRNA design and construction of CRISPR/Cas9 vector

The CHOPCHOP tool (https://chopchop.cbu.uib.no) was employed to choose two specific target regions within exons 14 and 15 of *CaPDS* located on chromosome 3. Subsequently, sgRNAs were designed based on various criteria, such as GC content, RNA folding, and potential off-target occurrences. The genome editing binary vector pKSE401 (Add gene, Catalog #62202),[36] which comprises a *Zea mays* codon-optimized Cas9 protein-coding gene controlled by a *2x CaMV 35S* promoter and *nptII* as a selection marker was used as the backbone for the cloning of the designed sgRNAs. The two designed gRNAs (gRNA1 and gRNA2) were subsequently inserted into the pKSE401 vector under the control of U6-26p and U6-29p promoters, respectively, using specific primer pairs (Table S2) (Fig. 1B).

2.4. Biolistic transformation and plant regeneration

The particle delivery complex comprising the plasmid containing *35S*: and *2X35S*:Cas9 was delivered into chilli pepper cotyledons using our standard particle delivery protocol.[37] Briefly, the plasmid DNA was precipitated onto 0.6 μm-sized gold particles (Bio-Rad, India) by mixing with 2.5 M $CaCl_2$ and 0.1 M Spermidine. Subsequently, the coated gold particles were centrifuged in a microfuge at 8,000 ×g for 30 s, and the supernatant was removed. The resulting pellet was then resuspended in 50 μl of sterile water. A volume of 10 μl of the precipitated plasmid DNA was loaded onto a macrocarrier and left to air dry. Approximately 350 cotyledon explants of 8–10 d were excised from axenic cultures and placed on 25 Petri dishes (12–15 cotyledons for each one) before being bombarded using a Particle Delivery System (PDS)-1000/He Gun (Bio-Rad, Hercules, CA) using standardized parameters.[37] A 9 cm flight distance, 900 psi pressure, 2 cm distance between the rupture disc and the macrocarrier were consistently deployed in bombardment. Subsequently, plant regeneration and selection were carried out in accordance with the methodology reported earlier.[35]

2.5. Identification of CaPDS gene edited mutant lines of chilli pepper

The leaf tissue (0.1 g) was collected from the kanamycin resistant, putative *Capds* mutant lines and was ground into a fine powder in liquid nitrogen. Following the cetyltrimethylammonium bromide (CTAB) extraction method,[38] genomic DNA was extracted and any RNA contamination was eliminated using the RNase and DNase-free kit (Roche Diagnostics GmbH, Indianapolis, USA). In the initial screening, the regenerated shoots were subjected to antibiotic selection with 80 mg/L kanamycin, followed by evaluation using *npt*II and Cas9 sequence-specific primers (Table S1). The integration of the T-DNA, encompassing the CRISPR/Cas9 assembly into putatively edited mutant lines was examined under the following conditions: an initial denaturation step at 95 °C for 3 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s, and a final extension cycle at 72 °C for 5 min. To detect mutations in the *Capds* edited lines, a DNA fragment spanning both targeted gRNAs was amplified a with specific primer pair (Table S1). The PCR amplicons were excised, and purified using the QIA quick gel extraction kit (Qiagen, USA). The purified products were then

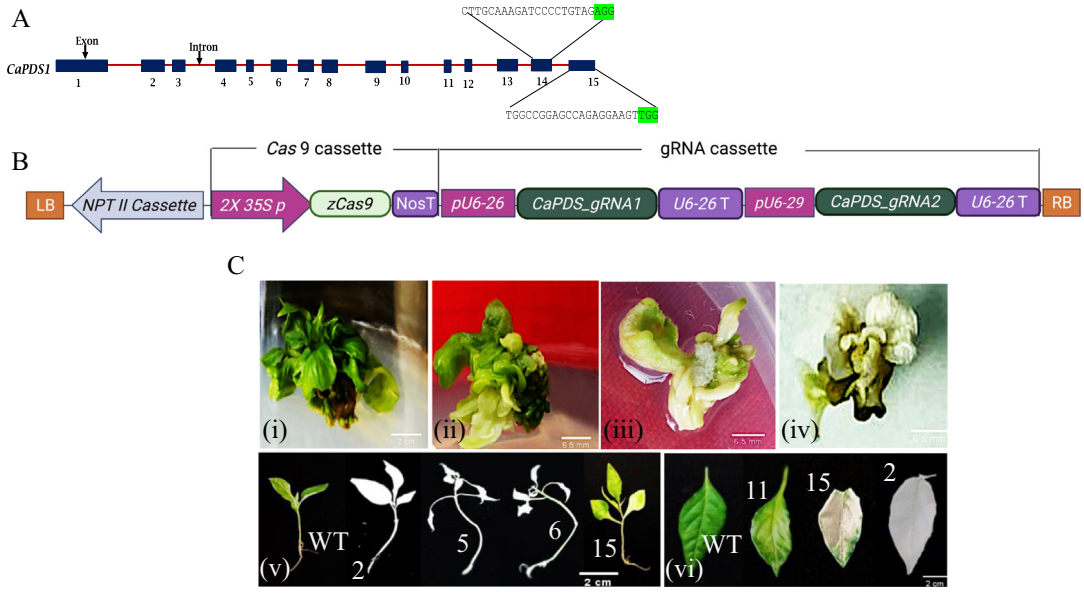


Fig.1. CRISPR/Cas9-mediated modification of *CaPDS* in Chilli pepper (*Capsicum annuum L*). **A**. Schematic representations of the targeted Chilli pepper *PDS* gene. The number of exons has been mentioned below each box representing an exons and red lines represent the introns. The two different gRNAs (gRNA1 and gRNA2) were designed from the exon 14 and 15 respectively. The PAM sequence was highlighted in green color. **B**. The CRISPR/Cas9 binary vector containing *2XCaMV 35S* promoter driven Cas9 protein employed for the stable biolistic transformation of chilli pepper. **C**. CRISPR/Cas9-mediated mutations of *CaPDS* in *in vitro* regenerated shoots of chilli pepper. Control Wild-type plants with completely green shoots. **(i),** edited albino shoots displaying a mosaic green and white shoot **(ii &iii)**, and fully albino shoots, **(iv)** cultured on selection media (MS salts + B5 vitamins + 0.5 mg/L TDZ + 0.2 mg/L IAA + 80 mg/L kanamycin). Phenotype of CRISPR/Cas9 based *CaPDS* mutations in 5 w old *in vitro* chilli pepper plantlets **(v)** and leaf from mosaic and albino plants **(vi)**. WT represents non-edited wild-type control plants with fully green tissues (leaf and shoots), 2,5, and 6 represents *Capds* edited lines showing full albino phenotype. 11 and 15 represents heterozygous *Capds* edited lines showing a mixture of green and white patches in leaves. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analyzed for DNA heteroduplex formation at the target region using a T7EI assay according to the manufacturer's instructions (New England Biolabs, USA) using the specified primer pair (Table S1). To detect indels in mutant lines, the pJET1.2 vector was cloned with purified PCR amplicons containing the target gRNAs (Thermo Fisher Scientific, USA). The indels were examined using the CLUSTALW and ExPASy tools (https: web.expasy.org/translate/).

2.6. Measurement of chlorophyll and carotenoid levels

To evaluate the efficiency of the CRISPR/Cas9 based targeted mutation and subsequent effect on chlorophyll and carotenoid production in chilli pepper *CaPDS* edited lines, we quantified these pigments as reported previously.[39] Briefly, fresh leaf tissues from *Capds* mutant lines and WT plants (20–30 mg fresh weight) were homogenized with 80 % acetone after being ground to a fine powder. The samples were then centrifuged at 10,000 rpm for 15 min at 4 °C to obtain a colorless supernatant, repeating the extraction process. The spectrophotometric measurement of the resulting supernatant was carried out using a UV-300 UV/Vis Spectrometer (Thermo Spectronic, USA) at specific wavelengths: 647 nm for chlorophyll-b (Chl b), 663 nm for chlorophyll-a (Chl a), and 450 nm for total carotenoid content. The estimations were determined using the previously reported formula.[40] The concentrations of carotenoid, Chl a, and Chl b were estimated and expressed in mg g^{-1} FW of extract.

2.7. Statistical analysis

The biochemical estimations were carried out in triplicate and repeated three times with similar results. Mean, standard error, and *t*-test values were calculated with respect to control and graphs were plotted using GraphPad Version 9.5.0.

3. Results and discussion

3.1. An efficient biolistic-delivered CRISPR/Cas9 based genome editing system established in chilli pepper

The nucleotide sequences of previously described *PDS* gene from different plants like tobacco,[41] soybean,[42] maize,[43] banana,[27] and tomato[44] were used as baits to search for homologous sequences within the reference genome of pepper in 'solgenomics'. Sequence alignment suggested that the *CaPDS* gene is aligned at the protein level indicating a conserved role in chilli peppers (Fig. S1). The *CaPDS* gene consists of 2198 bp of transcript sequence with 15 exons separated by 14 introns on chromosome 3 (Fig. 1A). To induce mutations in the *CaPDS* gene, two 23-base pair target sequences with a PAM site located at their 3′-ends were selected. These sequences were designed to target the fourteenth and fifteenth exons of the pepper *PDS* gene and were labelled as gRNA1 and gRNA2, respectively. The two distinct gene-specific gRNAs of *CaPDS* were inserted into the pKSE401:*Cas9*, which resulted in the pKSE401:*Cas9-CaPDS* construct (Fig. 1B).

One of the potential concerns with CRISPR/Cas9 system is its off-target effect.[45] To further examine the specificity of targeted *CaPDS* gene, off-target analysis was performed using different software such as CRISPR-OFFinder[46] and BLASTN program in the Pepper Genome of Sol Genomics Network, which did not detect any potential off-target mutations indicating high specificity for *CaPDS* gene editing. To optimize specificity, guide sequences should have a maximum number of consecutive mismatches to off-target sites, or at least three mismatches separated by fewer than four bases, at least two of which should be placed inside the PAM-proximal regions.[47] The designed gRNAs matched the majority of the aforementioned criteria, thereby enabling specific targeting (Fig. 1A).

This CRISPR/Cas9 vector was further bombarded into the cotyledons of chilli pepper cells using the biolistic delivery method.[37] The bombarded cotyledons along with control were transferred to the selective shoot induction medium.[35] The explants were subcultured at 12–14 d intervals, and the induced shoot buds proliferated after three subcultures (Fig. 1C). Subsequent subculture on the selective shoot elongation medium resulted in the proliferation of shoot buds turned into rosette structures (Fig. 1C). These rosette structured albino shoots were separated and subcultured on the same selective shoot elongation medium for two more weeks for further elongation. Subsequently, the elongated albino shoots were transferred to selective rooting medium containing 80 mg/L kanamycin.[35]

In recent times, several diverse approaches for delivering CRISPR/Cas9 reagents to plant cells ranging from *Agrobacterium* infection to nanoparticles have been concurrently developed.[48] However, despite these advancements, the frequent polyploidy and genome rearrangements, as well as the rigid cell walls make it challenging to introduce genome editing reagents into plant cells.[18] Although, *Agrobacterium*-mediated transformation may result in intact T-DNA delivery and single-copy events in different crops, it lacks consistency across species, and genotypes, particularly in more recalcitrant species like chilli pepper.[49] Intriguingly, Kota et al. (2019) have recently devised a biolistic delivery approach for effective chilli pepper chloroplast transformation. Furthermore, it has been shown that the particle bombardment approach may be used to introduce CRISPR reagents into squash (*Cucurbita pepo* subspecies pepo) cells.[33]Nevertheless, the use of biolistic methods to introduce CRISPR reagents into chilli peppers remains elusive.

In this study, we used biolistic protocol,[37] to deliver the CRISPR components into chilli pepper cells and transgenic mutant lines were recovered by following our previously established protocol.[35] As demonstrated in Fig. 1C, The albino phenotype is comparable to that of CRISPR/Cas-edited *PDS* mutants in other crops[23,26,50] indicating that *CaPDS* is effectively silenced by the biolistic delivery of pKSE401*: Cas9-CaPDS* construct into chilli pepper cells.

3.2. Molecular characterization of CRISPR/Cas9 induced mutations in CaPDS gene

A total of 21 independent mutant lines were recovered after selection on kanamycin-containing medium. Preliminary screening of regenerated plantlets using Cas9 coding sequence-specific and antibiotic (*nptII*) specific primers (Table S1) on isolated genomic DNA from transformed lines revealed that 16 of the examined lines carried the intact pKSE401*:Cas9-CaPDS* construct, demonstrating successful integration of the expression cassette into the genome (Fig. S2). The chromatogram-based sequence analysis (Fig. S3) revealed that transformants exhibiting visible phenotypes had mutated sequences in the *CaPDS* gene. Further, to detect the mutation in the targeted *CaPDS* gene, these transformed lines were subjected to T7 endonuclease I (T7E1) analysis. The T7E1 assay showed extra DNA fragments in edited lines (Fig. 2A). However, the remaining six edited lines did not undergo digestion by T7E1 endonuclease indicating a mutation efficiency of 62.5 % (Fig. 2B). Notably, three of the ten transformed lines (*Capds-2, Capds-5, and Capds-6*) exhibited albino phenotypes, whereas the other seven lines exhibited mosaic patterns with retarded growth, while the *in vitro* raised control plants showed no phenotypic alterations suggesting that the *CaPDS* function in the chilli pepper has been impaired.

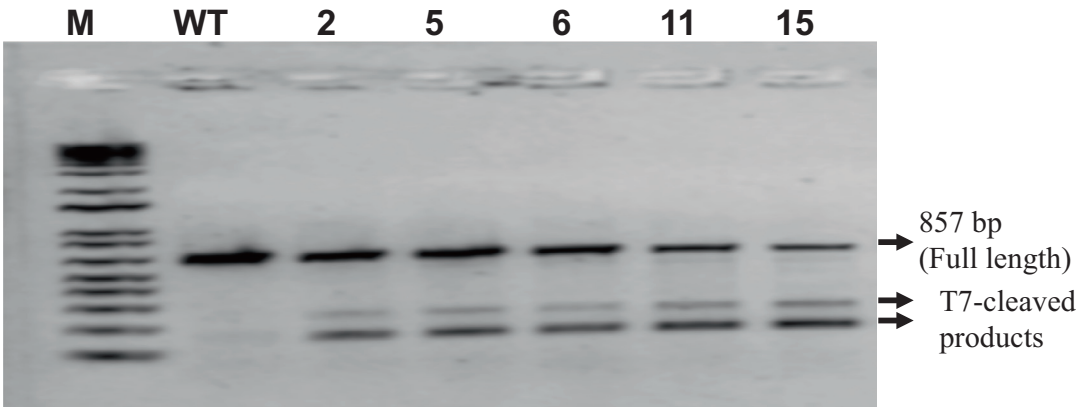
A phenotypic observation revealed that photobleaching occurred mostly in young leaves extending from the middle to the leaf tips (Fig. 1C). In addition, the mosaic leaves exhibited a succession of white and green patches, but the albino leaves were fully photobleached (Fig. 1C). The albino phenotype mutant lines *Capds-2, Capds-5, and Capds-6* were susceptible to light, and were more prone to die under greenhouse conditions in the soil, while plants with a mosaic phenotype survived and adapted to the soil conditions. Several previously published studies indicated that CRISPR/Cas9-mediated *PDS* gene-edited mutants were photosensitive and failed to survive.[23,32]

To further characterize the editing events in the transformants, an average of twelve clones were randomly selected from each of five distinct transformants (three albino and two mosaic phenotypes) for amplification and sequencing of the target region covering both gRNA1 and gRNA2 targets (Fig. S3). Sequence analysis revealed that the editing efficiencies of the two gRNAs were distinctive, and most deletions have occurred at the gRNA2 target region ranging from 11 bp to 24 bp (Fig. 2C). Single insertions or deletions (+/−1 bp) were the mutations that were most often seen in the edited lines. The *Capds-2, Capds-5, and Capds-6* albino mutant lines were found to be monoallelic and homozygous with larger deletions of −16, −24, and −18 bp at the gRNA2 target site, respectively. These findings suggest that a mutation in the *CaPDS* gene has a detrimental effect on the chilli pepper plant rendering the albino plants unable to thrive in soil. The albino mutant lines *Capds-2* and *Capds-6* had two distinct deletion patterns (Fig. 2C), whereas mutant lines *Capds−11* and *Capds−15* displayed heterozygous mutations with −12 and −18 bp deletions, respectively (Fig. 2C). The modifications, such as single nucleotide insertions and large deletions led to frame shift changes causing premature stop codons near the target region.[23] Consequently, this could produce a truncated CaPDS protein.

Notably, the genome editing efficiency in this study is significantly greater than that in hop (33.3 %),[23] carrot (35.3 %),[51] melon (42 %),[52] and strawberry (45 %)[53]. The variation in the efficiency of genome editing may be attributed to the activity of Cas9 endonuclease, the transformation method, and the explants used.[54,55] Although the *CaMV 35S*-driven CRISPR/Cas9 system targeting the *CaERF28* gene has been established in Capsicum, the rate of homozygosity has been varies, which may be attributed to Cas9 being driven by the constitutive promoter and *Agrobacterium* mediated transformation of hypocotyl explants.[56] Kim et al. (2020), highlighted the potential of genome editing in pepper (Sweet pepper cv, Dempsey and CM334) protoplasts derived from leaves or calluses by PEG-mediated delivery. Although they highlighted the efficacy of CRISPR-based genome editing tools in pepper protoplasts, their effectiveness in the heritability of the edited genes, as well as their comparative effectiveness (CRISPR/Cas9 Vs. CRISPR/Cas12a), are yet to be explored. Whereas Park et al. (2021), investigated three *Agrobacterium* strains, including AGL1, EHA101, and GV3101, to determine the most effective strain for pepper transformation.[57] Their findings revealed that GV3101 induced the highest number of Calli in sweet pepper Dempsey, implying its suitability for developing stable pepper transformation methods. Despite their success, they have not been able to recover stable genome-edited plants.[49,57]

In contrast, our findings indicate that both the *U6* and *2x CaMV 35S* promoters can effectively trigger a DSB at the *CaPDS* target site, achieving a genome editing efficiency of 62.5 % in the chilli pepper (Fig. 2B), which was on par with previous findings.[23,51] The editing efficiency of CRISPR/Cas9 may be increased by simultaneous targeting of two sites in the same gene. This would result in the deletion of the gene fragment between the two targeted sites, potentially enhancing editing efficiency.[58] However, the GC content of the target sites plays a role in the success of mutations in plants.[44] In our study, we used two sgRNAs with varying GC contents (gRNA1; 50 % and gRNA2; 65 %) to increase the possibility of producing loss-of-function mutations at distinct target sites within the *CaPDS* gene. Notably, the gRNA1 designed to target exon 14 was ineffective for site-specific mutation in the chilli pepper, but the gRNA2 targeted exon 15 was highly effective and had higher indel rates at the target site; this may be because of higher GC content set for the gRNAs. Therefore, these results reinforce the necessity of using multiple gRNAs concurrently in order to accomplish a successful gene knockout.[56,59] Furthermore, our findings align with prior

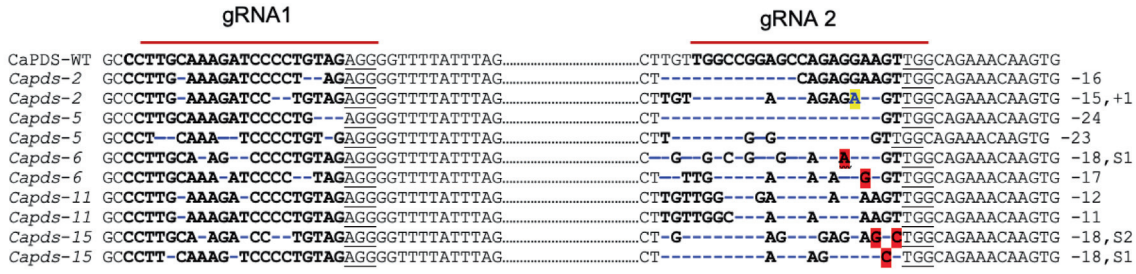
A

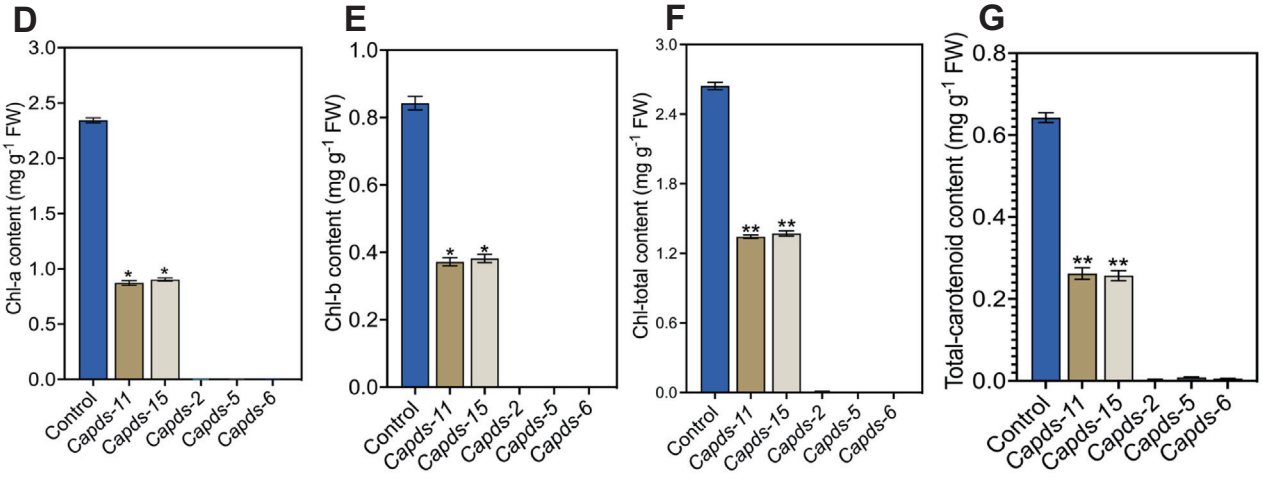


B

Plant lines analyzed#	Phenotype of transformants	No. of Plants	Mutation type (No. of plants)	Mutation efficiency (%)
	Albino	3	HM (2)	18.75
			HE (1)	
16	Mosaic	7	HM (0)	43.75
			HE (7)	
	Green	6	HM (0)	
			HE (0)	
Total		**16**	**10**	**62.5**

C





studies indicating that homozygous mutants were only produced in their first generation (T0) when two or more gRNAs were utilized to create cleavages at two adjacent target sites.[56,60]

3.3. CaPDS edited mutant lines exhibit dwarf albino phenotypes and reduced levels of carotenoids

The amounts of total carotenoid and chlorophyll in the leaves of WT and edited lines of chilli pepper were measured. Our analysis demonstrated a notable decrease in the essential photosynthetic pigments in the *CaPDS* edited lines compared to the control (WT) plants. The degree of albinism was proportionately correlated with the differing proportions of Chl a/b and carotenoid contents (Fig. 2D–G). These results were corroborated by previous studies suggesting that the *PDS* gene is essential for the biosynthesis of carotenoid, chlorophyll, and GAs.[26,28] In addition, all homozygous mutants with an albino phenotype exhibited reduced development when cultured *in vitro* (Fig. 1C). These findings further demonstrated that the *PDS* gene is not only important in plant carotenoid biosynthesis, but also a key target for herbicide inhibitors.[61] In fact, it has been demonstrated that the disruption of the *PDS* gene may result in a GA deficit. Due to the extreme sensitivity of *in vitro* organogenesis to hormonal balancing, it is important to do further work in this direction.

4. Conclusion

In conclusion, the protocol described here is a quick and very easy process that directly transforms totipotent tissues and organelles.[62] Additionally, the *CaPDS* knockout approach presented here generates clearly discernible mutant lines within 5–7 weeks. Due to the direct identification of the phenotype, confirmation of editing at the target site, it is not necessary for sequencing. The adoption of gene-editing technologies for the genetic improvement of chilli peppers with important traits such as improved nutrition, fruit quality, and resistance to pests and diseases may be greatly simplified with this suitable approach, which also offers a quick way to assess and validate CRISPR/Cas9 sgRNA targets. As an alternative, our particle bombardment-mediated transformation system enables the direct introduction of ribonucleoprotein (RNP) or CRISPR/Cas9 reagents into totipotent cells to develop transgene- free chilli pepper mutants.

CRediT authorship contribution statement

Mallesham Bulle: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Ajay Kumar Venkatapuram:** Writing – review & editing, Validation, Methodology, Investigation. **Sadanandam Abbagani:** Writing – review & editing, Supervision, Investigation. **P.B. Kirti:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Vijay Sheri, ICGEB, New Delhi, for assisting in experiments. Partial Financial support received from the Science and Engineering Research Board (SERB-DST), Government of India, New Delhi in the form of National Post-Doctoral Fellowship (N-PDF) (File No. PDF/2018/001226) to MB is gratefully acknowledged. PBK acknowledges the NASI-Senior Scientist Fellowship awarded by the National Academy of Sciences-India.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgeb.2024.100380.

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Fig. 2. Assessment of *CaPDS* gene edited chilli pepper plants. **A.** T7 endonuclease assay for confirmation of mutation in transformed chilli pepper lines. PCR amplified target from control (WT) and transformed lines (2, 5, 6, 11, and 15) were subjected to T7E1 assay. The edited lines showed fragmentation of target into two bands and corresponding bands were absent in the WT plants. M, 1 kb plus DNA ladder; WT, genomic DNA from wild type chilli pepper cultivar G4; 2, 5, 6, 11, and 15 represents *Capds* edited lines. **B.** Summarization of biolistic-delivered CRISPR/Cas9 based mutagenesis of the *CaPDS* gene in Chilli Pepper. Mutation events were confirmed after T7E1 assay. HM, Homozygous; and HE, Heterozygous. **C.** Sequence analysis of mutant chilli pepper plants. The target sequences of *CaPDS* are denoted in boldface and the PAM is indicated in the reference sequence for the wild type (WT). Below the WT sequence, sequences from each mutant line (*Capds-2, and Capds-5*, etc.) are shown. Substitutions are marked in red; insertions are highlighted in yellow, and deletions are represented by blue dashes. On the left side of the panel, the size and mutation type (insertion (+), deletion (−), or substitution (S)) are indicated. HM, Homozygous; and HE, Heterozygous *Capds* edited lines. **D.** Quantification of chlorophyll (Chl a), **E.** chlorophyll (Chl b), **F.** Total chlorophyll, and **G.** Carotenoid concentration in leaf tissue of *Capds* edited lines (Heterozygous lines, *capds-11,* and *capds-15;* Homozygous lines, *capds-2, capds-5, and capds-6*; Control, wild type chilli pepper cultivar G4). Pigment Concentration was calculated in mg/g of fresh weight. The student's *t*-test was used to assess the data in relation to the WT control to determine statistical significance. Statistically significant differences are indicated by an asterisk (*$p < 0.05$, **$p < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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