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# Demonstration of CRISPR-*cas*9-mediated *pds* gene editing in a tomato hybrid parental line

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**Abstract**

**CRISPR-*cas*9 mediated gene editing is a powerful tool proven for crop improvement and has great potential for incorporating novel traits into important genetic resources. In this study, *pds* gene editing in a parent line of a tomato hybrid by using CRISPR-*cas*9 system was successfully conducted. Three small guide RNAs (sgRNAs) were designed to target three exons of a tomato *pds* gene and were transformed along with a *cas*9 gene into the line of interest. Observation of chimeric albino phenotypes in the regenerated tomato shoots indicated the disruption of *pds* gene. Furthermore, expression of *cas*9 gene was determined by demonstration of its transcript accumulation in the primary tomato transformants. Sequence analysis of targeted *pds* exons showed point as well as deletion mutations in the sgRNA target sites. The result confirmed functional nature of the CRISPR-*cas*9 assembly used in the present study, which will enable incorporation of novel traits directly into the parents of tomato hybrids.**

**Key words:** Tomato, *pds,* CRISPR-*cas*9, sgRNA, gene editing

## Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops grown across the world. High yielding tomato cultivars are continuously being developed to meet the demand, however, the productivity is significantly affected by biotic and abiotic stresses. Hybrid tomato has shown yield advantage over varieties attributable to heterosis. Furthermore, growers have preferred hybrids over varieties due to fruit uniformity (Bai and Lindhout 2007). To maintain supremacy over varieties, parental lines of hybrids need to be bred for yield, fruit quality and resistance to diseases and pests (Cheema and Dhaliwal 2005). Various modern technology tools such as embryo rescue, DNA marker technology, have been applied to introgress various traits into parents of hybrids from wild and exotic germplasm (Shirasawa and Hirakawa 2013). During introgression of various traits from wild and exotic germplasm into parents of hybrids, breeders have encountered issues of genetic drag/ donor's genome content (DGC) (Stam 2003). It is not always possible to remove DGC from the recipient variety even after 5 to 6 backcrosses (Stam 2003; Semagn et al. 2006). This may result in the loss of original nature of tomato parent in which traits are aimed to be introgressed from wild or exotic species. This issue can be overcome by using more DNA markers for background selection. However, there are chances of retaining DGC in recipient variety if marker assisted selection is not complemented with phenotype selection (Stam 2003).

Complete DGC free gene transfer is possible through transgenesis and transgenic technology has enabled us to transfer gene/genes from any source to any crop plants (Visarada et al. 2009). Despite sizable adoption of the first generation of transgenic crops, there has been limited deregulation of GM traits in food crops. Tomatoes in particular have very few GM traits which have been commercialized (Clark et al. 2004; Bai and Lindhout 2007; De Steur et al. 2015). This bottleneck has compelled researchers to find alternative technologies that make precise alterations in the genome of the varieties or hybrids and, this may help to address regulatory hurdles to some extent

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(Wolt et al. 2015). GE (Gene Edited) crops hold promise, as genomes of many crop plants including tomato have been sequenced and functions of various genes have been extensively studied (Thottathil et al. 2016). In the last one decade, various engineered nucleases (EENs) have shown to create targeted variations in the plants genome (Wolt et al. 2015; Mujumdar et al. 2016; Osterberg et al. 2017). These EENs make double stranded breaks (DSB) or single stranded nicks (SSN) in the targeted region of the DNA. Repair of DSB or SSN in the DNA happens in non-homologous end joining (NHEJ) or homology driven recombination (HDR) manner. During this process, GE introduces all types of mutations; it could be of single base pair change, deletion, and substitution or additions (Belhaj et al. 2015; Alamalakala et al. 2016). Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-*cas*9 mediated genome editing system consists of single monomeric *cas*9 protein and chimeric RNA or short guide RNA (sgRNA). Sequence specificity of sgRNA is conferred by a 20 bp nucleotide sequence upstream to Proto-spacer Adjacent Motif (PAM) i.e. NGG site in the coding strand of target sequence (Quetier 2016). Two endonuclease domains, RuvC and HNH of *cas*9 make DSB, generally three bases upstream of PAM site. Here also, plant's endogenous system repairs the DNA mostly by NHEJ or in some cases by HDR (Bortesi and Fischer 2015). While, a *cas*9 variant, *cpf*1 recognized different PAM motif TTN and creates 5' overhang 15 to 23 bases way from PAM motif (Zetsche et al. 2015). Frequency of mutations by *cpf1* is reported to be higher compared to *cas*9 mediated DNA editing (Tang et al. 2017). CRISPR-*cas*9 mediated genome editing has been demonstrated in various crops (Ma et al. 2016), including horticulture crops such as Tomato (Brooks et al. 2014; Cermak et al. 2015; Pan et al. 2016; Soyk et al. 2017), Potato (Wang et al. 2015), Sweet Orange (Jia and Wang 2014), Apple (Nishitani et al. 2016) and Cabbage (Lawrenson et al. 2015).

Keeping pace with evolving market demands, it is imperative to edit key genes which can boost tomato productivity. Therefore, incorporating such edited genes in parental lines is a way to produce future tomato hybrids. Here, we demonstrate the CRISPR-*cas*9-mediated gene editing in a parent of a proprietary tomato hybrid by creating targeted knock-out mutations in phytoene desaturase (*pds*), an important gene in the carotenoid biosynthesis pathway.

## Material and methods

### *Single guide RNA design*

The *pds* gene sequence of *Solanum lycopersicum* L obtained from NCBI LOCUS X78271 and was verified in the parent of Mahyco tomato hybrid by PCR amplification. CRISPR-Plant, the university of Arizona online portal of CRISPR-*cas9* mediated editing, was used to design three sgRNAs targeting exon 2, 4 and 5 of the *pds* gene (NCBI X78271). Designed sgRNAs were selected based on minimal off target impact as shown by CRISPR-Plant bioinformatics tool.

### *Cas9/sgRNA vector construction*

Disruption of *pds* gene was planned using single SgRNA as well as multiple SgRNA strategies. *Streptococcus pyogenes* Cas9 gene sequence obtained from public domain was synthesized by GeneArt™, Gene synthesis Service, Thermo Fisher Scientific. The *Eco*RI and *Bam*HI restriction sites flanking to *cas*9 expression cassette (e35S Promoter-3X FLAG-N terminal NLS-*cas*9 gene-C terminal NLS-Nos terminator) were used to facilitate cloning into the plant transformation vector pCAMBIA 2300. Twenty bp DNAs expressing crRNA (targeting exon 2, 4 and 5 of *pds* gene) along with gRNA scaffold-polyT (Mali et al. 2013), driven by U6 promoter were synthesized by GeneArt™, Gene synthesis Service, Thermo Fisher Scientific. Synthesized U6-sgRNA-polyT cassette was cloned in pCambia2300-*cas*9 vector using *Sal*I- *Pme*I restriction sites (Fig. 1). The resultant binary vector

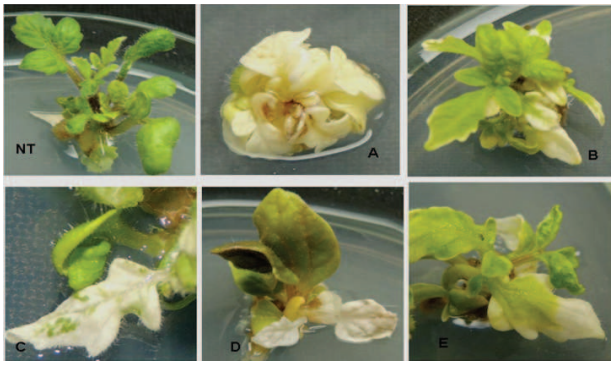


**Fig. 1. Albino phenotype in first generation ($T_0$) chimeric tomato shoots carrying *CRISPR-cas9* assembly targeting *pds* gene (NT-Non transgenic, Chimeric shoots -A, B, C, D, E)**

was mobilized into *Agrobacterium* strain EHA101 and used for tomato transformation.

### *Tomato transformation and plant regeneration*

Seeds of tomato were germinated on MS (Murashige and Scoog 1962) plant growth medium. Twelve days old cotyledons were cut from three sides and incubated with 0.2 O.D. of *Agrobacterium* carrying pCambia-Cas9-sgRNA binary vector. Furthermore, cotyledons were blotted on Whatman filter paper no. 42 (Sigma WHA1442042), transferred to MS medium with 0.8% agarose and 100 mM acetosyringone and; co-cultivated for three days at $22^0$C followed with three days post culture on MS medium with 0.5 mg/lit 2, 4, D. The tomato shoots were regenerated following three selections on MS with 1.5 mg/lit Zeatin, 50 mg/lit Kanamycin and 500mg/lit Cefotaxim. Regenerated tomato shoots were used for further molecular analysis.

### *Molecular analysis of T0 lines*

Regenerated tomato shoots were screened with PCR amplification of *cas*9 gene (Table 2 for PCR primer list) using following PCR conditions i.e. heat start at $95^o$C for 5 min followed with 35 cycles consist of $95^o$C for 30 sec, $58^o$C for 40 sec and $72^o$C for 1 min followed with 7 min incubation at $72^o$C. PCR positive shoots were used for T7 endonuclease1 assay to detect the mutation. PCR amplification of targeted region of pds exons 2, 4 and 5 were PCR amplified using following PCR conditions *i.e*., heat start at $95^o$C for 5 min followed with 35 cycles consist of $95^o$C for 15 sec, $50^o$C for 30 sec and $72^o$C for 30 min followed with 7 min incubation at $72^o$C. T7 endonuclease1 assay was performed as directed by kit manual (EnGen™ Mutation Detection Kit, NEB #E3321S). *pds* exons (2, 4 and 5) of selected tomato shoots that detects mutations by T7 Endo 1 assay and also shown chimeric albino phenotype were sequenced by Sangers di-deoxy method of sequencing.

### *Reverse transcriptase-PCR (RT-PCR) analysis of Cas9 gene*

To demonstrate the expression of *cas*9 gene at transcripts level, total RNA was extracted from cas9 PCR positive tomato shoots using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich #STRN50). First strand cDNA synthesis was carried out using 500ng of total RNA. The procedure was carried using SuperScript™ III First-Strand Synthesis System Kit (ThermoFisher #18080051). cDNA was used to perform PCR using following conditions i.e. $95^0$C for 5 min followed with 30 cycles consist of $95^0$ C for 30 sec, $72^0$C for 45 sec and $60^0$ C for 1 Min. PCR products were separated on 1.0% agarose gel and gel image were taken on gel documentation system.

## Results and discussion

Previous reports have demonstrated CRISPR-*cas*9 mediated inheritable genome editing in the model tomato cultivars, *viz*., M-82 and MicroTom and other horticultural crops (Brooks et al. 2014; Wang et al. 2015; Cermak et al. 2015; Pan et al. 2016; Soyk et al. 2017). We have demonstrated the *pds* gene editing in a parental line of a tomato hybrid by using crispr-*cas*9 system. The *pds* gene expresses an important enzyme of the carotenoid biosynthesis pathway (Parkhi et al. 2005). Disruption or silencing of this gene results in impaired carotenoid, chlorophyll and gibberellins biosynthesis and makes plants albino (Liu et al 2002; Qin et al. 2007). In this study, three 20 bp sequences with PAM on their 3' regions in *pds* gene locus were selected as sgRNA complementary sites, targeting exon 2, 4 and 5 with minimum off target effects (Table 1). Cas9 assembly along with sgRNA expression cassettes were transformed into tomato parental line.

**Table 1.** Short guide RNA target sequence of *pds* exon 2, *pds* exon 4 and *pds* exon 5 obtained by using CRISPR-Plant web tool

| Gene target | sgRNA target Sequence |
|---|---|
| *pds*Exon2 | GAGCTCGAGGTCGTCTTCTT**TGG** |
| *pds*Exon4 | GTCACAAACCGATACTGCTGG**AGG** |
| *pds*Exon5 | GATGGAGATTGGTACGAGAC**TGG** |

**Table 2.** Primer sequence of PCR primers

| Primer name | Sequence |
|---|---|
| *pds*Exon2 F | TTC TGA GGT TTG TGG ATC TT |
| *pds*Exon2 R | ACT TAT GAC CCA TTG ATT CG |
| *pds*Exon4 F | CTA AGC TGC CTT GAA CTT GT |
| *pds*Exon4 R | CCT ACC CCA AAA AGG ACT AC |
| *pds*Exon5 F | GAT TTG CAC GCT ATT TCT TC |
| *pds*Exon5 R | AAC GAT AAA CGA CAA ACG AG |
| Tomato *actin* F | TAT TGT GTT GGA CTC TGG TG |
| Tomato *actin* R | TGC TGG AAT GTG CTG AGA GAT GC |
| *cas*9 F | CTA CGA TGA TGA TCT CGA TAA CC |
| *cas*9 R | GAA CAA GAG ATC AAC GAT AGC CTT |

Putative transgenic tomato shoots were regenerated from the tomato transformation experiments in which binary vector carrying *cas*9 plus sgRNA 1 (exon 2) or sgRNA 2 (exon 4) or sgRNA 3 (exon 5) were used for

*Agrobacterium*-mediated transformation.

Pan et al. (2016) have demonstrated *CRISPR-cas9* mediated disruption of tomato *pds* gene and albino phenotype in tomato shoots of model cultivar Micro Tom, however, exons targeted for gene editing were not mentioned in the report. In agreement with this finding, the chimeric albino phenotype was also observed in some of the regenerated shoots in this study (Fig. 1) indicating the loss of PDS function. Complete or chimeric albino shoots were not developed into full grown tomato plants. Of 49 regenerated tomato shoots screened, 30 were found to be PCR positive for cas9 gene and were used for further expression and sequence analysis (Fig. 2). In the recent finding, functional nature of *cas*9 in the GE potato was reported

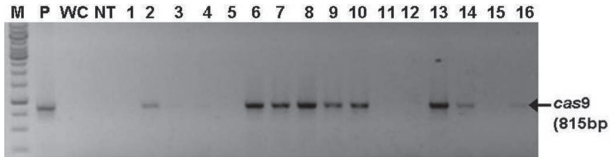


**Fig. 2. PCR amplification of 815bp *cas*9 gene in T0 tomato shoots carrying *CRISPR-cas9* gene assembly (M- 1 Kb DNA ladder, P-Plasmid Control, WC-Water control, NT-Non Transgenic control, 1-10: Putative T0 tomato shoots carrying CRISPR-*cas*9 gene assembly)**

by demonstration of transcript accumulation by RT-PCR and subsequent sequencing of target sequences (Wang et al. 2015). In this study, selected *cas9* shoots were analyzed for *cas9* transcript accumulation. Three of six PCR positive shoots tested for RT-PCR, showed *cas*9 transcript accumulation (Fig. 3). The RT-PCR

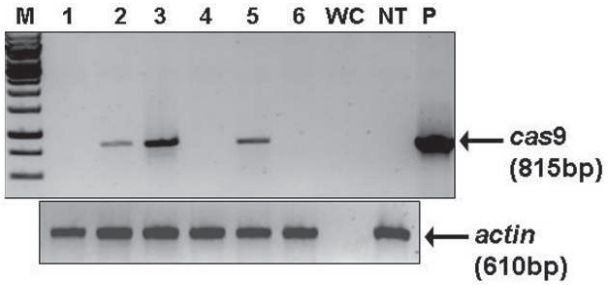


**Fig. 3. Demonstration of transcript accumulation in first generation transgenic tomato shoots carrying *CRISPR-cas9* gene assembly by reverse transcriptase-PCR (Samples: 1-6; WC-Water Control, NT-Non Transgenic; P-Plasmid Control)**

analysis of *cas*9 gene explains its functional nature in the first generation transgenic tomato shoots. A T7 endo-nuclease assay was used to detect mutations in edited samples (Pan et al. 2016). All 30 (PCR positive for *cas9*) shoots were analyzed with T7 endo1 assay by using PCR amplicons of *pds* exons 2, 4 and 5 that contains sgRNA target sites (Fig. 4). Three

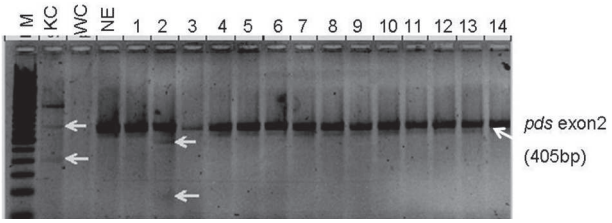


**Fig. 4. Detection of mutation in exon 2 of *pds* gene by T7 Endonuclease assay using EnGen™ Mutation Detection Kit (M-50bp DNA ladder, KC-Kit Control, WC-Water Control, NE-Non Edited tomato plant, 1-14: tomato shoots carrying CRISPR-*cas*9 gene assembly)**

amplicons (two of exon 2 and one from exon 5) showed mutations by T7 endo1 assay. To know the exact location and nature of the mutations detected by T7Endo 1, two chimeric shoots were sequenced. One shoot, TBM-3-55 showed point mutation near PAM site in exon 5 while other one, AGT-6 showed deletion mutation in exon 2 where three bases were deleted four bases next to PAM site. One more mutation detected beyond 20 base sgRNA site in the exon 2 (Fig. 5). This showed repairing through NHEJ following


**Fig. 5. Multiple Sequence Alignment of SgRNA target site of tomato pds gene by CLUSTALW web tool**

sgRNA directed Cas9 cleavage at the desired target sites. In the first generation of transgenic plant, obtained mutations could be of mono-allellic homozygous, biallelic homozygous or it could be in heterozygous in nature (Wang et al. 2015). Since the selected shoots were chimeric albino, it is most likely

that it contains edited as well as unedited alleles. This could be the reason that all clones from the same sample did not show the mutations as PCR amplicons might have carried edited or unedited mixture of heterogenous DNA.

These results demonstrate that the CRISPR-Cas assembly is efficient in generating targeted mutations in the first generation of transgenic tomato plants. The present study provides a proof of concept and validates new traits development using *CRISPR-cas9* system in the other crops of farmer's inertest. Since the desired traits can be directly developed into the parents of the hybrids, breeders can save time on introgression of the traits by using marker assisted backcross breeding and issue of DGC during backcrossing can be avoided.

**Authors' contribution**

Conceptualization of research (VP, AB, LA, VM, BC); Designing of the experiments (VP, AB); Contribution of experimental materials (BC); Execution of field/lab experiments and data collection (RP, VG, BP); Analysis of data and interpretation (SC, VP, RP); Preparation of manuscript (VP, AB, BC).

**Declaration**

The authors declare no conflict of interest.

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