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ORIGINAL ARTICLE

CRISPR/Cas9-mediated efficient editing in *phytoene desaturase* (*PDS*) demonstrates precise manipulation in banana cv. Rasthali genome

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Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) has been reported for precise genome modification in many plants. In the current study, we demonstrate a successful mutation in *phytoene desaturase* (*RAS-PDS*) of banana cv. Rasthali using the CRISPR/Cas9 system. Two *PDS* genes were isolated from Rasthali (*RAS-PDS1* and *RAS-PDS2*), and their protein sequence analysis confirmed that both PDS comprises conserved motifs for enzyme activity. Phylogenetic analysis of RAS-PDS1 and RAS-PDS2 revealed a close evolutionary relationship with other monocot species. The tissue-specific expression profile of *RAS-PDS1* and *RAS-PDS2* in Rasthali suggested differential regulation of the genes. A single 19-bp guide RNA (gRNA) was designed to target the conserved region of these two *RAS-PDS* and transformed with *Cas9* in embryogenic cell suspension (ECS) cultures of cv. Rasthali. Complete albino and variegated phenotype were observed among regenerated plantlets. DNA sequencing of 13 plants confirmed the indels with 59% mutation frequency in *RAS-PDS*, suggesting activation of the non-homologous end-joining (NHEJ) pathway. The majority of mutations were either insertion (1–5) or deletion (1–4) of nucleotides near to protospacer adjacent motif (PAM). These mutations have created stop codons in *RAS-PDS* sequences which suggest premature termination of RAS-PDS protein synthesis. The decreased chlorophyll and total carotenoid contents were detected in mutant lines that revealed the functional disruption of both *RAS-PDS* genes. Our results demonstrate that genome editing through CRISPR/Cas9 can be applied as an efficient tool for banana genome modification.

Keywords Banana transformation · Carotenoid · Embryonic cell suspension · Genome editing · Mutation

Introduction

Targeted genome editing (GE) is a modern approach for genome modification, and its application has been reported in various organisms. GE can be utilized for understanding the function of a gene or developing new trait for the crop improvement (Ronald 2014; Nogué et al. 2016; Scheben et al. 2016; Wolt et al. 2016a, b). Engineered endonucleases such as zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) have emerged as tools for GE. These nucleases respond in a target-specific manner and induce a double-strand break (DSB) in the gene sequence (Osakabe and Osakabe 2014). The DSB is repaired either by homologous recombination (HR) or non-homologous end-joining (NHEJ) mechanisms. NHEJ is error-prone repair mechanism and usually creates insertion or deletion (indel), which may lead to disruption of gene function (Bortesi and Fischer 2015). Among these tools, CRISPR/Cas9 is reported as more efficient in targeting the specific region of the gene sequence (Nishitani et al. 2016; Pan et al. 2016). It requires a set of two molecules, endonuclease (Cas9 protein) and single-guide RNA (sgRNA) and therefore acts as a host-independent gene-targeting platform. The targeting efficiency achieved by CRISPR/Cas9 has been observed up to 70% in

Sequence data from this article have been deposited in the GenBank data libraries under accession numbers: *RAS-PDS1* (MF574096) and *RAS-PDS2* (MF574097)

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maize (Char et al. 2017) and zebrafish (Albadri et al. 2017). The off-targeting of CRISPR/Cas9 is low due to the selection of specific and unique sgRNA and requirement of protospacer adjacent motif (PAM) sequence for Cas9 activity. The application of the CRISPR/Cas9 system has been demonstrated in many plant species for development of noble agronomic traits (Wolt et al. 2016a, b; Miao et al. 2013; Zhang et al. 2014), resistance to herbicides (Noman et al. 2016; Chen et al. 2017) and diseases (Wang et al. 2014; Peng et al. 2017).

Phytoene desaturase (PDS) is one of the rate-limiting enzymes in carotenoid biosynthesis pathway (Cazzonelli and Pogson 2010; Bai et al. 2016). It catalyzes the desaturation of colorless phytoene into ζ-carotene, which further converted into lycopene, a colorful compound in the carotenoid biosynthesis pathway (Fig. 1). The carotenoid biosynthesis pathway interacts with multiple metabolites such as abscisic acid (ABA), strigolactones, gibberellic acid (GA), phytol chain of chlorophyll, plastoquinones, and tocopherols as shown in Fig. 1. The ABA and strigolactone hormones regulate the development and assist plants to adapt to different environmental conditions (Xie et al. 2010; Ruiz-Sola and Rodríguez-Concepción 2012; Havaux 2014; Hou et al. 2016). Hence, the deformation or knockout of *PDS* gene affects the photosynthesis and carotenoid biosynthesis that subsequently lead to albinism and retarded growth of the plant (Tian 2015; Nishitani et al. 2016). The *PDS* gene has been used as a marker for the demonstration of GE in several plant species. For instance, it has been edited through CRISPR/Cas9 approach in *Arabidopsis thaliana* (Feng et al. 2013), *Nicotiana benthamiana* (Jiang et al. 2013; Li et al. 2013a; Nekrasov et al. 2013; Upadhyay et al. 2013), *Oryza sativa* (Feng et al. 2013), *Nicotiana tabacum* (Baltes et al. 2014), *Citrus sinensis* (Jia and Wang 2014), *Populus tomentosa* (Fan et al. 2015; Zhou et al. 2015), *Medicago truncatula* (Meng et al. 2017), and *Petunia* hybrid (Zhang et al. 2016).

Banana is ranked fourth after rice, wheat, and maize in the list of global food crops (Perrier et al. 2011; Dash and Rai 2016). It also ranks at second position next to mango for the most consumed fruit in the world (Dash and Rai 2016). Though, it is least genetically improved relative to other major crops due to its complex genetic makeup. The application of conventional breeding for genetic improvement in banana is difficult due to the ploidy level and nature of parthenocarpic fruit development (Dash and Rai 2016; Kaur et al. 2016). Thus, the application of innovative biotechnological approaches, such as GE, could be considered as a promising strategy in banana

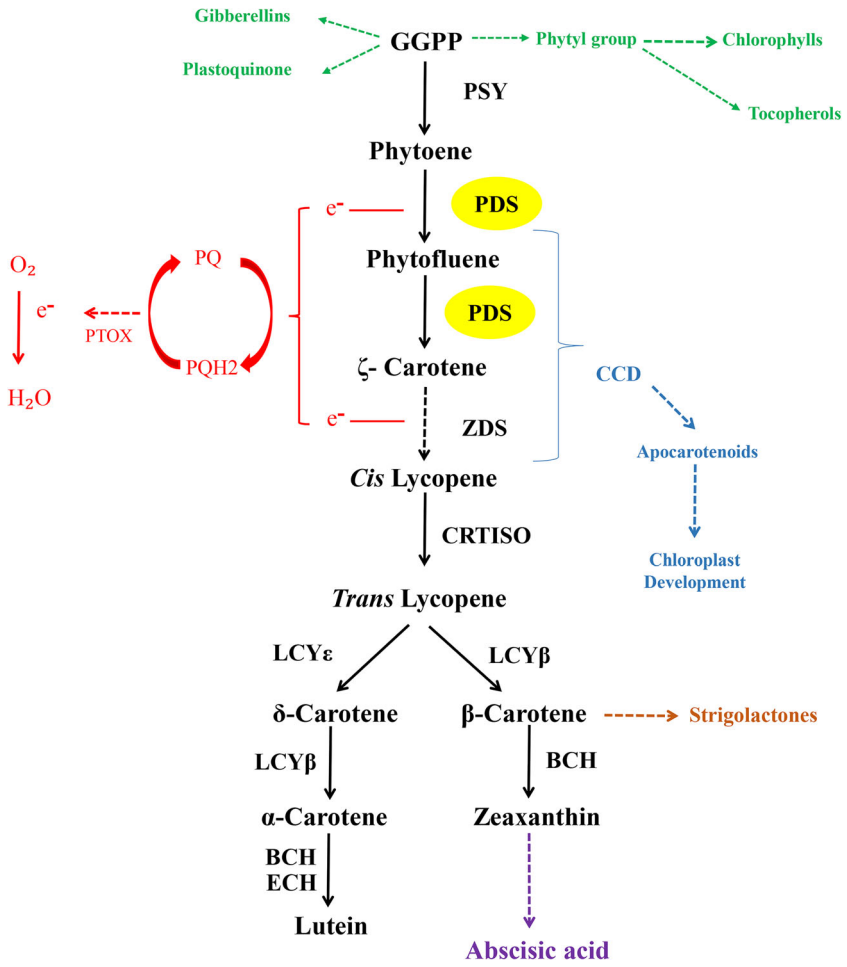

Fig. 1 Schematic pathway of carotenoid biosynthesis. Enzymatic reactions are represented by arrows; dashed lines represent multiple enzymatic steps. Enzymes: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYβ, lycopene β-cyclase; LCYε, lycopene ε-cyclase; CRTISO, carotenoid isomerase; BCH, β-ring hydroxylase, ECH, ε-ring hydroxylase; CCD, carotenoid cleavage dioxygenase. Compounds: GGPP, geranylgeranyl diphosphate; PQ, plastoquinone; PQH_2, plastoquinol; PTOX, plastid terminal oxidase

improvement programs. In the present study, we demonstrate the establishment of the CRISPR/Cas9 system in banana by precise targeting of the *PDS* gene in cv. Rasthali (AAB) genome. *PDS* gene was chosen from carotenoid biosynthesis pathway due to its characteristic perceptible affirmation. This study is substantiated by sequencing analysis of the target region of *PDS* gene and phenotypical assessment of mutants with a decrease in chlorophyll and total carotenoid contents. Hence, our analysis revealed that the CRISPR/Cas9 system could serve as a useful tool to facilitate studies of gene function and can also be exploited in the banana improvement programs.

Material and methods

Plant materials

Different plant tissues and immature flower buds of cv. Rasthali were collected from the banana germplasm field of the National Agri-Food Biotechnology Institute (NABI), Mohali. Fruit fingers, bract, pseudostem, root, leaf, and unripe and ripe fruit-pulp tissues were frozen immediately in the liquid nitrogen and stored at − 80 °C till further use. Immature male flower buds were used to isolate male flowers as the source of explant for the preparation of embryogenic cell suspension (ECS) culture and regeneration of plants by following previously described methods (Côte et al. 1996; Shivani et al. 2017).

Isolation, cloning, and sequence analysis of Rasthali *PDS*

The whole genome sequence of banana (*Musa acuminata*) was downloaded from the Banana Genome Hub database (D'Hont et al. 2012; http://banana-genome.cirad.fr/) and used for the mining of the *PDS* gene. Annotated *PDS* sequences of maize (*Zea mays*), rice (*Oryza sativa*), and arabidopsis (*Arabidopsis thaliana*) were used for BLAST analysis in banana genome database to identify similar sequences. The *PDS* sequence was retrieved and used for designing primers for the isolation of full-length open reading frame (ORF) of *PDS* from Rasthali (Table S1).

Total RNA was isolated from leaf tissue of Rasthali as per previously described method (Kaur et al. 2017). Ambion DNase I (RNase-free) (Thermo Fisher Scientific, USA) was used to eliminate DNA contamination. The cDNA was prepared by using RevertAid First Strand cDNA Synthesis Kit from 2.5 μg of DNA-free total RNA following the manufacturer's instructions (Thermo Fisher Scientific, USA). The full-length ORF of *PDS* was amplified and named *RAS-PDS* gene. The amplicon was ligated into the pJET1.2 vector (Thermo Fisher Scientific, USA) and confirmed by sequencing using 3730xl DNA analyzer (Applied Biosystems, USA).

The percentage identity of the RAS-PDS protein sequence was determined with respect to maize, rice, and arabidopsis. The motif analysis was performed as per method described by Li et al. (2013b). The Compute pI/MW tool on the ExPASy server was used for predicting the theoretical isoelectric point and molecular weight (Bjellqvist et al. 1994; http:// web.expasy.org/compute_pi/). The phylogenetic tree was constructed using the MEGA6.0 tool (Tamura et al. 2013). The protein sequence of RAS-PDS and PDS from different monocot and dicot species were used for the phylogenetic tree analysis. The neighbor-joining method was used to infer evolution. The bootstrap value was set to 1000.

Differential expression analysis of *PDS* in Rasthali

To check the expression of two identified putative *RAS-PDS*, the gene-specific primers were designed to perform quantitative real-time PCR (qRT-PCR) analysis. Total RNA was isolated from different tissues (fruit fingers, bract, pseudostem, root, leaf, and unripe and ripe fruit-pulp) of cv. Rasthali. The cDNA was prepared, and qRT-PCR study was carried out as previously described method (Kaur et al. 2017). The *Actin1* (GenBank Accession No. AF246288) gene (banana housekeeping gene) was used as an internal control. The data was estimated in terms of relative fold expression following the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). The primers used in the study are given in Table S1.

Construction of genome editing module for *RAS-PDS*

To test whether the CRISPR/Cas9 system can be used to precisely edit an endogenous gene in banana, we designed a specific gRNA from the most conserved region (5th exon) of the *RAS-PDS* genes (Fig. 2). A 19-bp gRNA sequence was selected as a target site by using the SSFinder tool (Upadhyay and Sharma 2014) and confirmed by manual analysis (Table S1). The 19-bp sequence had tri-nucleotide PAM sequence, i.e., 5′-AGG-3′ on its 3′ end. This gRNA sequence was inserted into binary vector pRGEB31 (Xie et al. 2014) under rice *snoRNA U3* promoter using *Bsa*I restriction site (Fig. 2). This vector harbors *Cas9* endonuclease-encoding sequence under dual *CaMV 35S* promoter as well as *neomycin phosphotransferase* (*NPTII*) and *hygromycin phosphotransferase* (*HPT*II) selection marker genes. The vector prepared was named as CRISPR_RAS_PDS (Fig. 2). Kanamycin and hygromycin were used for the selection of positive bacterial clones and transformed plants, respectively.

Agrobacterium-mediated transformation

ECS culture was developed by following the earlier reported method (Shivani et al. 2017). The embryogenic nature of ECS was confirmed by using microscopic (Leica, Germany) observation and used for the transformation. The CRISPR_RAS_PDS construct was transformed into *Agrobacterium tumefaciens* strain

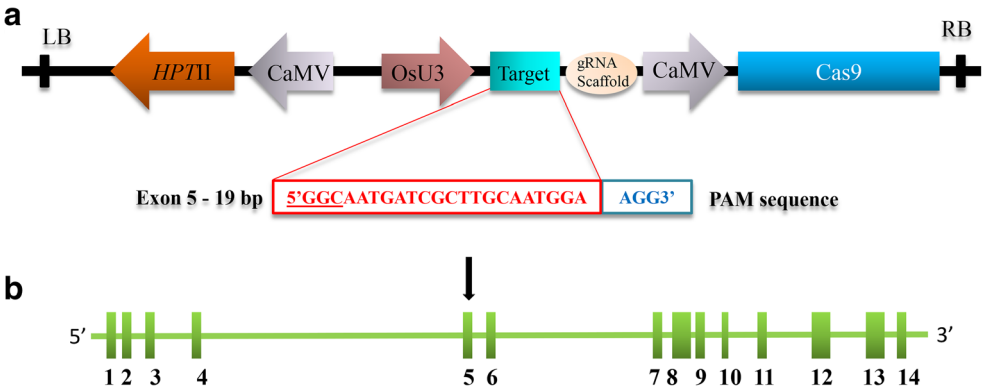

Fig. 2 Schematic map of CRISPR/Cas9 construct for the *RAS-PDS* editing. **a** Modified CRISPR/Cas9 vector named as CRISPR_RAS_PDS. **b** The position of gRNA for targeting the *RAS-PDS* sequence. Boxes indicate exons, numeric digits indicate exon number, and line indicates the intron

Agl1 by electroporation using a Gene-Pulser device (Bio-Rad, USA) and named as Agl1_PDS. ECS transformation with Agl1_PDS and regeneration of transformants were performed as described previously (Khanna et al. 2004) with some modifications. Briefly, a single colony of Agl1_PDS was inoculated in 5 ml of Luria broth (LB) primary culture medium containing rifampicin (50 mg/l) and kanamycin (50 mg/l) and grown at 28 °C for 2 days at 200 rpm. One milliliter of primary culture was transferred to fresh 50 ml yeast mannitol broth (YMB) medium containing the same composition of antibiotics and grown at 28 °C for 1 day at 200 rpm. The culture was centrifuged at 5000 rpm, and the pellet was resuspended in 50 ml induction medium containing acetosyringone (200 μM). The bacterial suspension was incubated at 25 °C for 3–4 h at 90 rpm to an OD_{600} of 0.5–1.0. The ECS was given 5 min heat shock and used for infection. The infected ECS was transferred on to co-cultivation medium for 3 days at 23 °C in the dark. After co-cultivation, ECS was rinsed with cefotaxime (400 mg/l), blotted to remove the excess bacterial suspension and placed on a semi-solid medium containing cefotaxime (250 mg/l) for 15 days. The embryogenic cells were subcultured onto fresh regeneration medium containing cefotaxime (250 mg/l) and hygromycin (20 mg/l). At least five rounds of selection, each for 2–3 weeks were performed for the recovery of transformed cells. Healthy cells were then transferred to germination and subsequent rooting medium supplemented with cefotaxime (200 mg/l) and hygromycin (30 mg/l) till the development of plants. The empty vector (pRGEB31) was transformed into Rasthali ECS and used as a control. All in vitro cultures were incubated in plant tissue culture chambers (Percival, USA).

Genomic DNA extraction, screening, and confirmation of editing using sequencing

Genomic DNA was isolated from the putative *RAS-PDS* mutant and plants using DNeasy Plant Mini Kit (Qiagen, Germany) as per manufacturer's instruction. Target and *Cas9* sequence-specific primers (Table S1) were used for screening the transformants. *RAS-PDS* specific primers (Table S1) were used for amplification of the target region (311 bp). The amplified target region was cloned into the pJET1.2 vector using a CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA). From each transformant, 5–8 clones were sequenced using 3730xl DNA analyzer (Applied Biosystems, USA) to check any modification in the target gene.

Pigment extraction and measurement

Fresh leaf tissues from *RAS-PDS* edited, and control plants were used for pigment extraction (Sumanta et al. 2014). The leaf tissue (0.5 g fresh weight) was grinded, and the extraction was carried out using methanol. The sample was centrifuged at 10,000 rpm for 15 min at 4 °C. Extraction was repeated until colorless supernatant obtained. The sample was diluted and measured absorbance at 470 nm, 653 nm, and 665 nm using spectrophotometer (UV-2700 UV-VIS Spectrophotometer, Shimadzu, Japan). The concentration (μg/ml) of chlorophyll a, chlorophyll b, and total carotenoid was calculated according to the following equations

$$\text{Chl a}^{*} = (16.72\ A_{665} – 9.16\ A_{653})$$
$$\text{Chl b}^{*} = (34.09\ A_{653} – 15.28\ A_{665})$$
$$\text{TC}^{*} = (1000\ A_{470} – 1.63\text{Chl a} – 104.96\text{Chl b})/221$$

In equations, A = *absorbance*, Chl a = *chlorophyll* a, Chl b = *chlorophyll* b, TC = *total carotenoid*

Statistical analysis

All experiments were performed in triplicates. The data of mutant lines in pigment quantification experiment was compared to the control plant. The fold change of genes expressed in different tissues was calculated relative to the lowest expressing gene in the tissues. The data are presented in mean ± SD and statistically analyzed by Student's paired *t* test. The statistical significance was cheeked at $P \leq 0.05–0.0001$ ($^{*}P \leq 0.0001$) with respect to control/the lowest expressed gene in a tissue. The Prism GraphPad software Ver. 5.01 (GraphPad Software, Inc. USA) was used for statistical analyses.

Results

RAS-PDS sequence analysis

Single *PDS* gene was (GSMUA_AchrUn_randomT12680_001) identified in banana genome by using known PDS protein sequences from the maize, rice, and arabidopsis. Two *RAS-PDS* (*RAS-PDS1* and *RAS-PDS2*) genes were isolated from Rasthali. The sequences of *RAS-PDS1* and *RAS-PDS2* were submitted to NCBI database under accession number MF574096 and MF574097, respectively. RAS-PDS proteins were similar in length (572 amino acids) and showed 97% similarity at sequence level. The deduced size of the two RAS-PDS proteins was comparable (RAS-PDS1, 64.59 kDa and RAS-PDS2, 64.51 kDa). The theoretical isoelectric point of RAS-PDS2 (8.32) was higher than RAS-PDS1 (8.14). Both, RAS-PDS (1 and 2) showed 77–80% protein similarity with respect to the PDS of maize, rice and arabidopsis (Fig. 3a). RAS-PDS proteins had three motifs, i.e., the dinucleotide binding motif, putative carrier motif, and carotenoid-binding domain (Fig. 3a). The evolutionary relationship was inferred using different PDS protein sequences from various monocot and dicot species (Fig. 3b). The result showed the categorization of RAS-PDS1 and RAS-PDS2 with monocot plant species. PDS proteins from dicot plants clustered into a different clade.

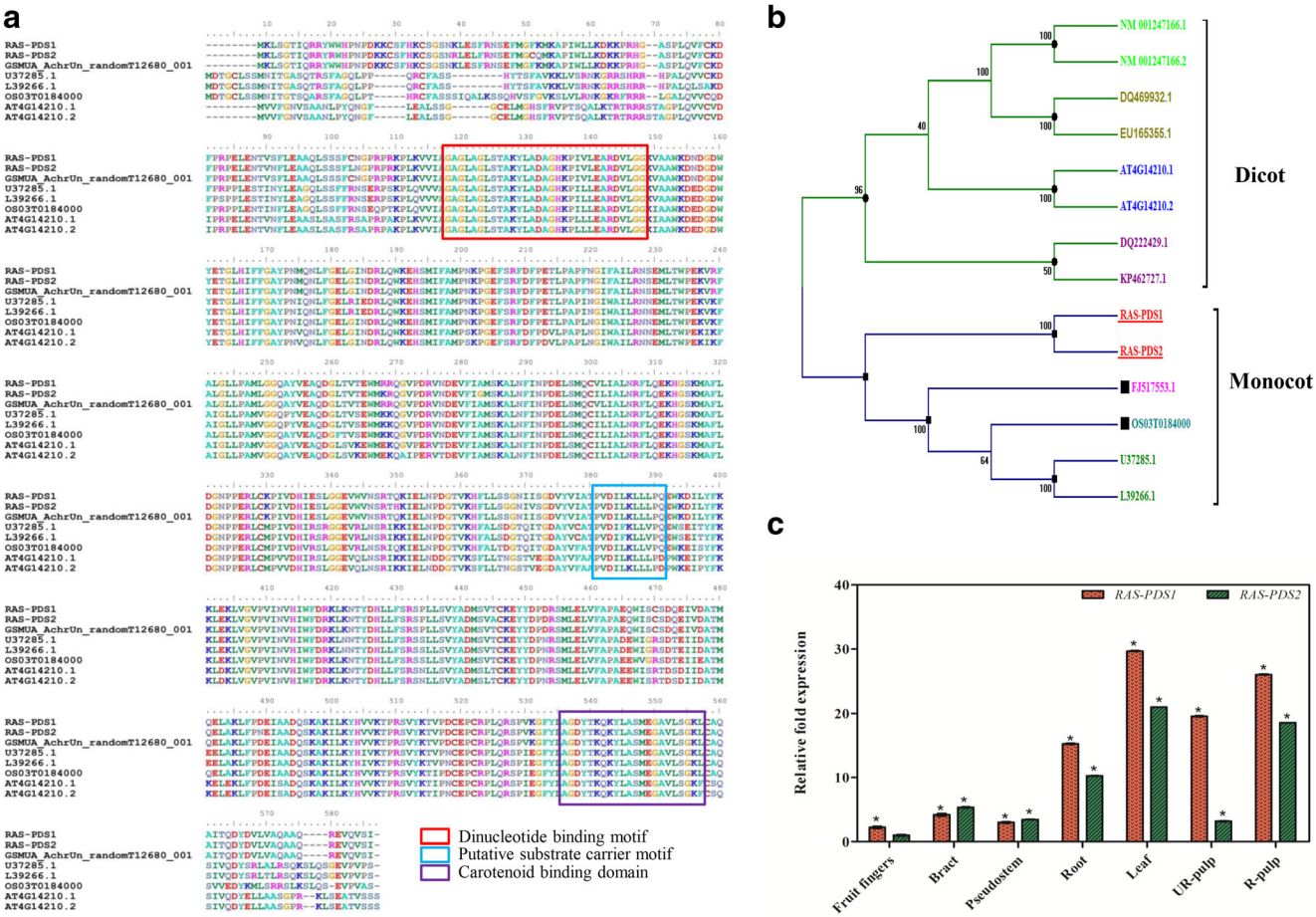

Fig. 3 Sequences analysis of RAS-PDS. **a** Multiple sequence alignment of RAS-PDS1, RAS-PDS2 with its homologs from banana genome database (GSMUA_AchrUn_randomT12680_001), *A. thaliana* (AT4G14210.1, AT4G14210.2), *Z. mays* (U37285.1, L39266.1), and *O. sativa* (OS03T0184000). Colored boxes on amino acids indicate the position of motifs and domain. **b** Phylogenetic tree of PDS proteins. Neighbor-joining based phylogenetic tree of the deduced PDS protein sequences of Rasthali (RAS-PDS1, 2) inferred with different plant species. GenBank accession numbers of PDS used are from *S. lycopersicum* (NM_001247166.1, NM_001247166.2), *D. carota* (DQ222429.1), *C. maxima* (KP462727.1), *N. benthamiana* (DQ469932.1, EU165355.1), *T. aestivum* (FJ517553.1), *A. thaliana* (AT4G14210.1, AT4G14210.2), *Z. mays* (U37285.1, L39266.1), and *O. sativa* (OS03T0184000). **c** Spatial distribution of *PDS* homeologs in Rasthali. Transcript expression profiles are presented in fruit fingers, pseudostem, bract, root, leaf, UR-pulp (unripe pulp), and R-pulp (ripe pulp). The gene expression was normalized with *actin1* taken as internal control. Bars denote mean fold expression as compared to the lowest expression of the gene in the group of tissues ± SD. Statistical analysis was performed using Student's paired *t* test. Statistical significance was checked at $P \leq 0.05$–0.001 and the symbols on the top of bars represent significance level ($^*P \leq 0.0001$) with respect to lowest expressing gene (*RAS-PDS2*) in the fruit fingers

Tissue-specific expression analysis of *RAS-PDS*

Expression analysis of two *RAS-PDS* genes in different tissues of Rasthali was performed. The fold change of genes expressed in different tissues was calculated relative to the *RAS-PDS2* expression (lowest gene expression) in fruit fingers. The differential expression pattern of *RAS-PDS1* and *RAS-PDS2* was observed in different tissues (Fig. 3c). In general, the higher expression of *RAS-PDS1* was detected at varying levels in different tissues except for the bract. The lowest expression of both homologs was observed in fruit fingers, whereas the highest transcript accumulation was found in the leaf tissue (Fig. 3c).

Transgenic development and screening

Microscopic visualization of callus comprised of somatic embryos and their respective suspension cells (ECS) are shown in Fig. S1. Globular and translucent somatic embryos were visualized on the surface of callus (Fig. S1a). The suspension cells used for transformation were spherical and contained dense cytoplasm (Fig. S1b, c). Transformed embryogenic cells were regenerated and germinated on hygromycin-containing selection media. The transformed cells harboring the T-DNA encompassing *HPT*II gene survived on the selection media (Fig. S1d). The non-transformed cells were unable to grow on selection media and eventually turned brown and died. Total 70 hygromycin-resistant plantlets with CRISPR_RAS_PDS construct were recovered from the germination medium while only 22 of these rooted. All 22 putative mutant plants were screened for the presence of target and *Cas9* sequence by PCR. The positive plants showed amplification of the ~ 1468 bp fragment with the target and *Cas9* sequence-specific primers (Fig. S2).

Phenotypic observation of transformants

Putative mutant plants had slower germination growth (Fig. 4a) with respect to control plants (Fig. 4b) on the selection medium. Most of the transformants showed complete albinism (Fig. 5b–e) and did not survive on the rooting medium. In variegated phenotype, leaves showed either small streaks or spots in early stages and later on chimeric phenotype having both green and albino phenotype was observed (Fig. 5f–h). Plants showing variegated phenotype (Fig. 5f–h) survived and acclimatized in the soil pots.

Genotyping of transformants

All PCR-positive plants were subjected to detect the mutation (deletion and insertion) in *RAS-PDS* genomic sequence. A 311-bp fragment from the genomic DNA accompanying the target region was cloned and sequenced (Fig. S3). Sequencing results confirmed mutations in 13 out of 22 plants, while the control plant did not show any changes in the *PDS* sequence. Thus, the mutation efficiency obtained in *RAS-PDS* was 59%. We noted insertion (7 plants), deletion (5 plants), and both insertion and deletion (1 plant) in different mutant lines adjacent to PAM region (Fig. 6). Three type of insertion patterns such as the addition of 5 nucleotides in 3 plants (16-line, 37-line and 63-line), 2 nucleotides in 2 plants (54-line and 59-line), and 1 nucleotide in 2 plants (41-line and 42-line) were observed (Fig. 6 and Fig. S4). Similarly, four types of deletion patterns, i.e., deletion of 38 nucleotides in 62-line, 4 nucleotides in 08-line, 2 nucleotides in 44-line and 52-line, and 1 nucleotide in 60-line were observed. A single mutant plant (46-line) showed both insertion and deletion of 5 and 1 nucleotides, respectively. These mutations lead to the formation of a stop codon due to frameshifting in the gene sequence (Fig. 6 and Fig. S5).

Quantification of chlorophyll and carotenoids content

Chlorophyll and total carotenoid content were quantified in leaflets of mutant lines. The content of chlorophyll and carotenoids was significantly lowered in mutant plants as compared to the control plants (Fig. 7). Mutant plants with different degree of albinism showed different levels of chl a, chl b, and carotenoids (Fig. 7 and Fig. S6).

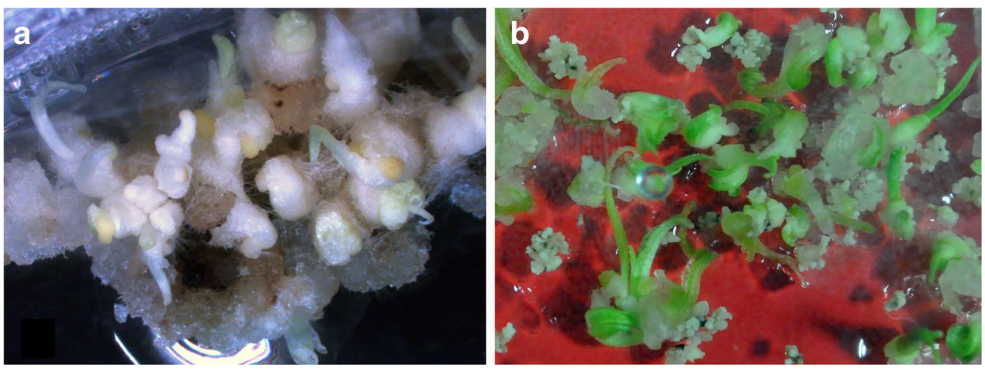

Fig. 4 Observation of the *RAS-PDS* mutants growth on the germination medium. **a** Slow growth and albinism exhibited in transformed embryos. **b** Healthy growth of control (empty vector) embryos

Fig. 5 Phenotypic observation of transformed plantlets. **a** Control plant. **b**–**e** Complete albino plants. **f**–**h** Variegated phenotype of *RAS-PDS* mutants

Discussion

CRISPR/Cas9 is recognized as the most efficient genome editing tool in various organisms including several plant species. Its application has been reported in arabidopsis (Li et al. 2013a), tobacco (Nekrasov et al. 2013; Li et al. 2013a), wheat (Upadhyay et al. 2013), maize (Liang et al. 2014), soybean (Cai et al. 2015; Jacobs et al. 2015; Michno et al. 2015; Sun et al. 2015), tomato (Čermák et al. 2015; Pan et al. 2016), potato (Butler et al. 2015; Wang et al. 2015), *Populus* (Fan et al. 2015; Tingting et al. 2015; Zhou et al. 2015), apple (Nishitani et al. 2016), and petunia (Zhang et al. 2016). These reports demonstrated that the CRISPR/Cas9-based mutagenesis is target specific in plants.

Here, we successfully demonstrated genome editing in *PDS* of banana cv. Rasthali using the CRISPR/Cas9 system. Previously, we showed variation in the coding sequence of *phytoene synthase* (*PSY*) genes within banana cultivars (Kaur et al. 2017). The CRISPR/Cas9 system works in a very precise and target-specific manner. Hence, it is desirable to select a unique target region for *PDS* gene editing. We isolated two *PDS* from Rasthali (*RAS-PDS1* and *RAS-PDS2*) and performed their in silico characterization. A single *PDS* was retrieved from banana cv. DH-Pahang having "AA" genotype (Banana

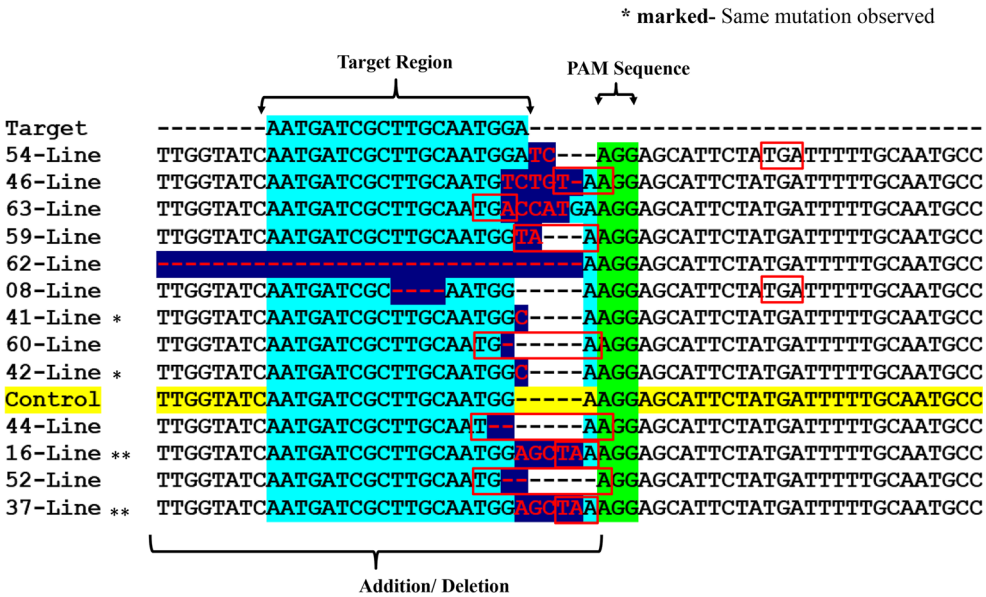

Fig. 6 Sequence analysis of *RAS-PDS* mutant plants. Cyan-colored sequence shows the target region. The yellow line represents control *RAS-PDS* sequence. The sequence with green background shows protospacer adjacent motif (PAM). Red-colored letter in the blue background and red dashes in the blue background indicate the insertion and deletion of nucleotides, respectively. Red boxes indicate the newly formed stop codons leading to premature termination of protein synthesis. In lines with asterisks (*, **), similar mutations were observed

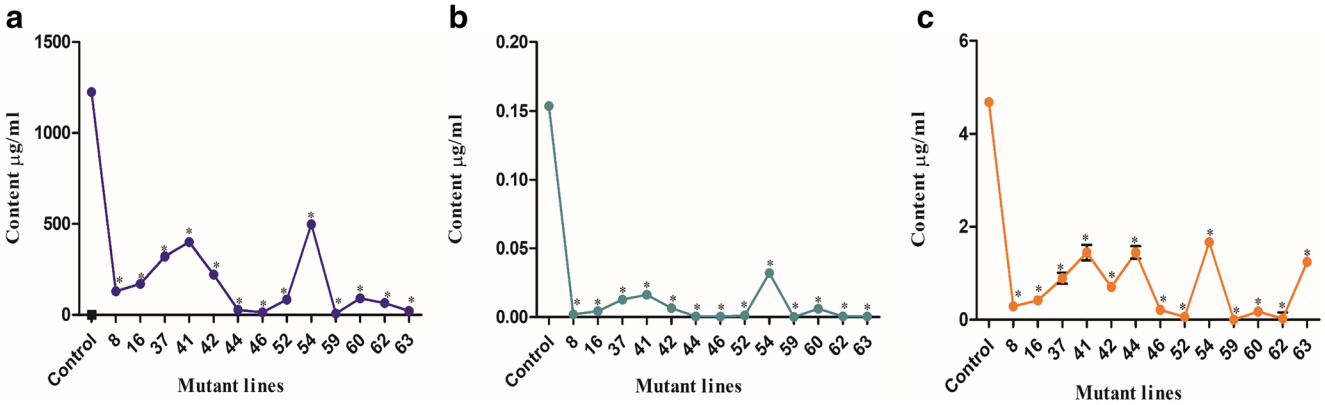

Fig. 7 Pigment quantification in leaf tissue of mutant lines. **a** Chlorophyll a. **b** Chlorophyll b. **c** Total carotenoid estimation. The data are presented in mean ± SD and analyzed by Student's paired *t* test. The data of each experiment was compared to control (empty vector plant) for the determination of significance ($*P \leq 0.0001$). Graphs were prepared using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The spectrum observed is given in Fig. S6

Genome Hub), whereas two *PDS* genes were identified from Rasthali. These two genes could be homeologs because Rasthali has two types of genomes, i.e., "A genome" and "B genome" (AAB). The functional protein encoded by *PDS* should include the dinucleotide binding motif, putative carrier motif, and carotenoid-binding evolutionarily conserved domains which are responsible for catalytic activity (Li et al. 2013b). Both RAS-PDS1 and RAS-PDS2 deduced sequences contain these conserved domains. We noted dinucleotide binding motif at the N-terminal of both PDS sequences which are a characteristic signature of all known carotenoid desaturases (Pecker et al. 1992; Li et al. 2013b). Further, expression analysis of both *RAS-PDS* ensures functionality of both homeologs in Rasthali. Hence, the conserved region of two identified *RAS-PDS* homeologs was selected for efficient targeting in Rasthali genome. A similar approach was reported in wheat for efficient editing of *Mildew-Resistance Locus* (*MLO*) homeologs (Wang et al. 2014).

PDS regulates biosynthesis of carotenoids which are an essential component of photosynthetic machinery. The disruption of *PDS* gene function results in albino phenotype and growth abnormalities in the plant. Previously, *PDS* has been selected as a model gene for establishing RNAi (arabidopsis; Wang et al. 2005), TALENs (maize; Liang et al. 2014; Char et al. 2015, soybean; Du et al. 2016), and CRISPR/Cas9 (petunia; Zhang et al. 2016) approaches in different plants. We observed complete albino as well as variegated phenotypes with lowered carotenoid and chlorophyll depositions in leaves of *PDS* mutant banana plants (Fig. 7). Our results are congruent with studies carried out in other plants such as apple (Nishitani et al. 2016), tomato (Pan et al. 2016), tobacco (Gao et al. 2015), and arabidopsis (Qin et al. 2007). The variegated phenotype observed in our study might be governed by multiple factors. For instance, genome heterozygosity, single/biallelic modifications, or functional efficiency of gRNA/Cas9 complex in the banana genome could be the possible factors for these variations. The selected banana cv. Rasthali is a triploid (AAB); hence, the triallelic mutation is required for homozygous genetic modification. We also found complete albino plants, but most of them did not survive during tissue culture process. The PDS executes two-step desaturation reactions that converts phytoene to ζ-carotene (Cazzonelli and Pogson 2010; Ruiz-Sola and Rodríguez-Concepción 2012). Desaturation is linked with redox chain reactions for electron transport which is involved in the production of quinones, plastid terminal oxidase (PTOX), and molecular oxygen (Carol et al. 1999; Yu et al. 2007; Yu et al. 2014). PTOX has a crucial role in the greening process, and lack of PTOX results in variegated phenotype due to bleaching of chloroplast in the absence of carotenoid (Carol et al. 1999; Rumeau et al. 2007). Hence, we anticipate the complete obstruction of RAS-PDS function has affected electron transport chain which enhanced release of free radicals and subsequent death of these mutant plants. Few of these plants were acclimatized with abnormalities (deformed leaves, albino/variegated leaves, and stunted growth), though being unable to survive for a long time suggested the high possibility of homozygous mutation of *RAS-PDS* gene. Such phenotypic observations are reported in other plant species, i.e., watermelon (Tian et al. 2017), apple (Nishitani et al. 2016), tobacco (Gao et al. 2015), rice (Shan et al. 2013), and arabidopsis (Qin et al. 2007). All mutant plants showed slower growth as compared to the control (Fig. 4). This could be due to the disturbance in carotenoid and gibberellic acid biosynthesis pathways that hampered growth and development of these plants (Qin et al. 2007). We identified three types of mutation in Rasthali plants such as insertion, deletion, and both addition and deletion. The similar type of mutations has also been reported in apple (Nishitani et al. 2016) and arabidopsis (Feng et al. 2014). In the current study, two mutant lines (41-line and 42-line) showed similar mutation pattern suggesting that they might have arisen from the single transformed cell line (Fig. 6). All the edited lines showed frameshift mutation that leads to the formation of the stop codons. It suggested termination of PDS protein synthesis and subsequently resulting in photo-bleaching.

In different plant species, a range of mutation frequency is reported using CRISPR/Cas9 approach. For instance, in maize 70% (Char et al. 2017) and rice 85.4% (Ma et al. 2015), mutation efficiency is reported. The mutation efficiency from 50 to 60% was observed in tomato (Brooks et al. 2014; Pan et al. 2016) and soybean (Li et al. 2015); similarly, we also found 59% mutation efficiency in *RAS-PDS* in banana cv. Rasthali genome. The optimized CRISPR/Cas9 tool presented here can be utilized towards studies of gene function and modification of certain genes that may be related to nutritional and/or agronomical important characteristic of banana. However, considering banana as a vegetatively propagated crop, further improvement of CRISPR/Cas9 approach can lead to the development of the transgene-free banana crop. The transgene-free technology can shorten the way through legislative, legal regulations to the market approval and thus easy acceptance of the product (Wang et al. 2016; Wolter and Puchta 2017). Recently, transient expression of CRISPR/Cas9 in the protoplast of wheat (Kim et al. 2017) and direct delivery of CRISPR/Cas9 ribonucleoprotein (RNP) with appropriate gRNA to the protoplast of fruit (grape and apple) and food (bread wheat) crop plants (Malnoy et al. 2016; Zhang et al. 2016; Liang et al. 2017) have shown efficient targeted mutagenesis. This has opened an opportunity to generate non-transgenic plants, but success in the regeneration of stable plants from genome-edited protoplasts is still a challenge. Hence, this approach needs to be established for the production of the successful transgene-free crop. Taken together, the present work laid a foundation for the elucidation of the functional aspect of the CRISPR/Cas9 system in banana and could be the step forward in the direction to generate genetically improved banana plants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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