



Application of CRISPR/Cas9 genome editing system for molecular breeding of orchids

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SUBMITTED 24 January 2020 REVISI 17 April 2020 ACCEPTED 27 May 2020

ABSTRACT Orchid is an important ornamental plant in Indonesia due to their natural beauty of flowers. In the tropical forest, orchids are being acquired for trading and commercial market. Thus, the effort is required to proliferate orchid in large quantities for conservation and improve the floral variation for plant breeding. The purpose of this study is to develop a firm methodology of molecular breeding of orchids using CRISPR/Cas9 KO system. The plant material used was *Phalaenopsis amabilis* protocorms growth on NP medium+pepton (2 g/L). Protocorm were submerged in the culture of *Agrobacterium tumefaciens* that Ti-plasmid had been filled with a T-DNA construct of a pRGE32 vector harboring sgRNA with *PDS3* sequence. Detection for transformants was confirmed by PCR using HPT primers (545 bp), Cas9 primers (402 bp), PDS primers (280 bp) and trnL-F (1200 bp) as an internal control. The results showed that 0.96% PDS transformants were obtained from *PDS3T2* lines. Several transformant showed pale leaf color compared to non-transformant plants. This study suggests that the target gene has successfully edited by CRISPR/Cas9 system and could be applied for that functional gene editing in orchids.

KEYWORDS CRISPR/Cas9; Genome editing; Molecular breeding; Orchid; Phytoene desaturase (PDS)

1. Introduction

Orchid (Familia: Orchidaceae) is an important horticultural plant in Indonesia. Some orchids, such as *Dendrobium* and *Phalaenopsis*, are known as popular potted ornamental plants for their beautiful and long-lasting flowers (Tong et al. 2019; Cai et al. 2015). On the contrary, the shooting emergences of orchids in term of seedling production are difficult to achieve naturally and the flowering time is very time consuming. These conditions are common problems in orchid breeding. Therefore it is necessary to overcome those limitations in order to promote and accelerate the seedling production and flowering time. Those problems could be solved through CRISPR/Cas9 genome editing system to edit shooting and flowering genes. CRISPR/Cas9 is the latest genome editing technology that has been already used in various animals and plants including model and non-model ones. With its high efficiency, CRISPR/Cas9 could be used for targeting biallelic genes simultaneously (Tsutsui and Higashiyama 2016).

Conventional genetic engineering strategy has several

issues and limitations, one of which is the complexity associated with the manipulation of large genomes of higher plants. However, creation of novel tools for breeding and biotechnology, an application area of genetic engineering, has received significant focus resulting in accelerated development of useful tools. At the present, gene editing techniques have the potential to substantially accelerate plant breeding.

Genome editing is a group of technologies that give scientists the ability to change an organism's DNA (Hsu et al. 2014). Targeted genome editing using artificial nucleases has the potential to accelerate basic research as well as plant breeding by providing the means to modify genomes rapidly in a precise and predictable manner. The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system is a new developed tool for the introduction of site-specific double-stranded DNA breaks (Liu et al. 2017). Cas9 protein induces double-strand breaks (DSBs) (Shen et al. 2017), which then repaired by the intracellular system. These technologies allow genetic material to be added, removed, or altered at particular locations in the genome,

it can be edits the form of point mutation, insertion, and deletion through the non-homologous end-joining (NHEJ) pathway (Chiruvella et al. 2013) or by gene replacement through homology-directed recombination (HDR) (Hahn et al. 2018).

This research objective is to develop CRISPR/Cas9 method for use in orchid in order to produce desirable allele in less than 20 months compared to the conventional breeding that need several years. In this preliminary study, we used Phytoene desaturase (*PDS3*) gene which is a rate-limiting enzyme in carotenoid synthesis. The *PDS3* gene converts phytoene to colored ξ -carotene in a two-step desaturation reaction (Wang et al. 2009). *PDS* gene encoding the phytoene desaturase enzyme is involved in carotenoid biosynthesis pathway (Naing et al. 2019), therefore mutations in those genes cause albino phenotype (Koschmieder et al. 2017). In general *PDS3* gene is widely used as a common marker gene in many plant species due to its ease of detection. Based on the easy detection of mutation phenotypically, this gene was chosen as target for genome editing. This is a preliminary study to find out how high mutation frequencies can be obtained with the CRISPR/Cas9 genome editing system in *P. amabilis* orchids, which can be further applied to edit other functional genes in orchids.

2. Materials and Methods

2.1. Plant Materials

Mature *P. amabilis* capsules were sterilized with 75% ethyl alcohol, 0.1% (w/v) natrium hydrochloride, then washed with sterile distilled water. After sterilization, the capsule was opened by cutting it in half longitudinally, then the seeds were sown on NP + peptone media (2 g/L). Cultures were maintained in an incubator with a temperature of day/night $\pm 25^\circ\text{C}$, orchid seeds then grew into a protocorm (developing orchid embryo). In general, protocorms could be used as targets because of their high regeneration capacity.

2.2. Vector Construction and Targets Selection

The vector pRGE32 in *Agrobacterium* strain EHA105 was used to transfer the T-DNA which contain CRISPR/Cas9 into orchid plant (Figure 1). The T-DNA

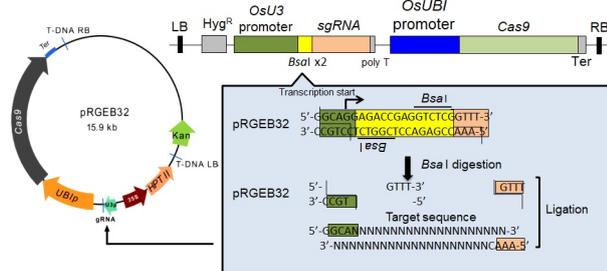


FIGURE 1 Plasmid pRGE32 construction containing T-DNA carried CRISPR/Cas9 (Xie et al. 2015).

region of this vector harbors hygromycin phosphotransferase (*Hyg^R*) as the selection marker genes. *Hyg^R* gene was driven by the CaMV 35S (35S) promoter and ended by the 35S' terminator, and cloned into the T-DNA in the pRGE32 vector. *Agrobacterium*-mediated transformation was conducted according the protocol described by Semiarti et al. (2010, 2011) for genetic transformation into plant.

To create a genome edited *P. amabilis* by using CRISPR/Cas9 system, an RNA-guided genome editing vector line pRGE32 (*Bsa* I)-Cas9 was used for expressing engineered sgRNA and Cas9 in cells. The sgRNA expression cassette was driven by the U3 promoter. This promoter was a small nuclear RNA (snRNA) promoter that initiates transcription at an adenine nucleotide and drives the high expression of sgRNAs (Shan et al. 2013; Ma et al. 2015). Based on homology search by BLAST, we found mRNA sequence of *PDS3* from *Phalaenopsis equestris* orchid (XM_020730635). It is 1767bp from AUG to UAG in length, and total length of mRNA in database is 2176 bp (Figure 2A). The protein motive showed that there is Pf01593 amino oxidase protein family that might be important to function the phytoene desaturase enzyme in carotenoid biosynthesis pathway (Naing et al. 2019). The DNA sequences of *P. amabilis* were selected as the candidates to determine the NGG as the protospacer adjacent motifs (PAM). To study the efficiency of CRISPR/Cas9 genome editing in *P. amabilis*, we selected two targets in *PHYTOENE DESATURASE 3 (PDS3)* gene. *PDS3* is involved in carotenoid synthesis in which the mutation shown albino phenotype (Qin et al. 2007). Twenty nucleotides were selected from the nucleotide sequence no 312-346 region of the *PDS3* genes. Target 1 (*PDS3T1*) and target 2 (*PDS3T2*) were separated by 9 bp. The sequence of *PDS3T1* is TTCGTGACTGTTTACGTCCC and *PDS3T2* sequence is CTAGAAGTGGAATTGCTGG (Figure2B).

The specificity of these candidates are examined by BLAST search against the genome sequence of *P. amabilis*. We checked it by BLAST and found *P. amabilis* *PDS3*mRNA is expected to be almost identical to that of *P. equestris* based on our sequence data (Figure 2B). In order to make the CRISPR/Cas9 constructs, two DNA oligonucleotides were chemically synthesized for each gRNA. The two oligonucleotides were complementary for the corresponding sequence to the spacer, and the sequences GGC and AAAC were added to the 50 end of the forward and reverse oligonucleotides, respectively, to allow the formation of cohesive ends of *Bsa*I restriction sites following annealing (Kui et al. 2017).

2.3. Selection of Transformant Protocorms and Plant Regeneration

After three days of co-cultivation and transformation process have done, the infected protocorms were transferred to selection medium containing 10 ppm hygromisin and incubated for 4 weeks. After that, survived protocorms that indicated transformant candidates with green color were transferred to NP medium with NAA: BA (1:2). Af-

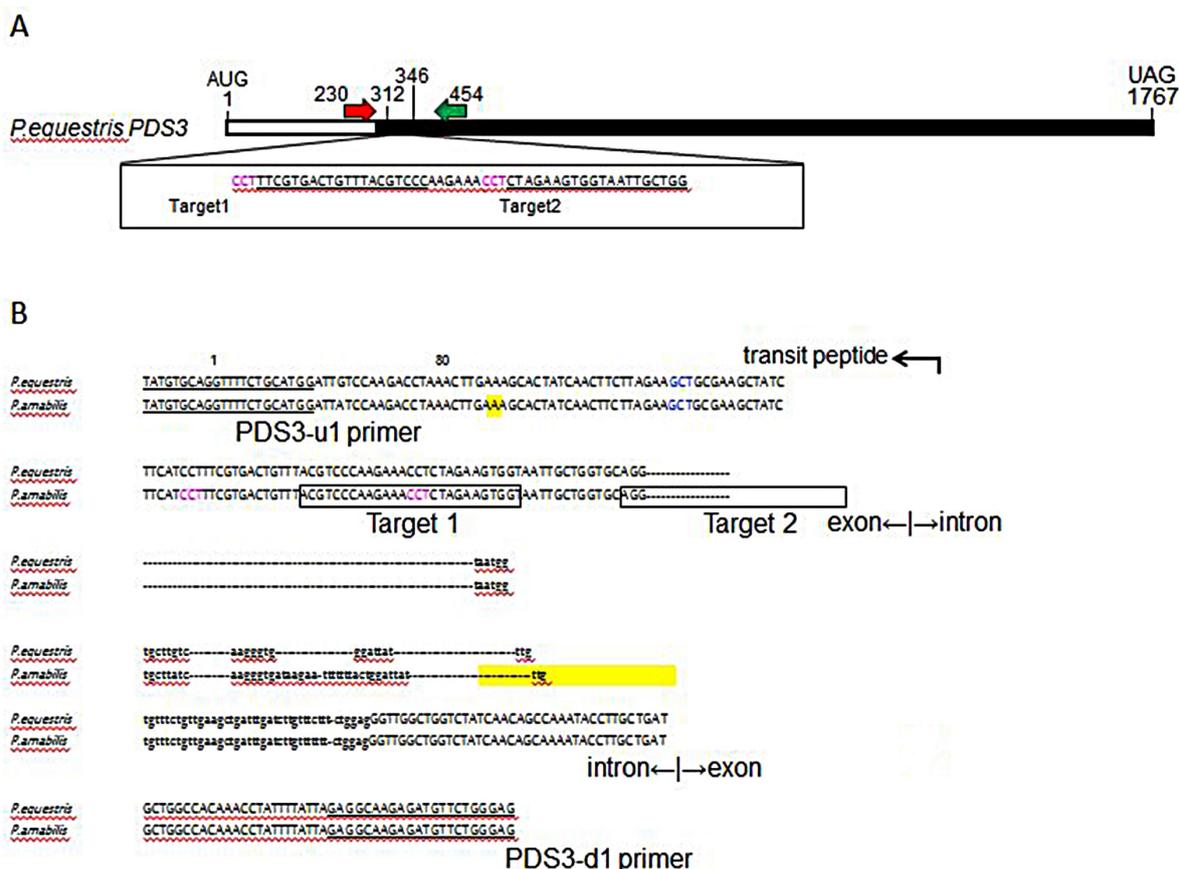


FIGURE 2 Map of PDS3 gene of *P. equestris* and target sequence position for CRISPR/Cas9 Genome editing system. (A) PDS3cDNA of *P. equestris* based on PDS3mRNA sequence from database; (B) PDS3 gene structure alignment of *P. equestris* and *P. amabilis* genom DNA. Red arrow: PDS3-u1 forward primer, green arrow: PDS3-d1 reverse primer, blue box: Predicted protein family region.

ter protocorms grown on the medium, molecular analysis was carried out to see the integration of T-DNA in plant genomes.

TABLE 1 List of primer sequences used to amplify CRISPR target region.

Primer	Primer Sequences (5' to 3')	Amplicon size
HPT	R: 5' AGGCTATGGATGCGATCGCTG F: 5' TCGGACGATTGCGTCGCATC	545 bp
Cas9	R: 5' TTACTTTTTCTTTTTTGCCTG F: 5'CACCATGGACTATAAGGACCACGAC	402 bp
PDS	R: 5'CTC CCA GAA CAT CTC TTG CCT C F: 5' TAT GTG CAG GTT TTC TGC ATG G	280 bp
Trn1-F (Internal control)	R: 5' ATTTGAACTGGTGACACGAG F: 5' CGAAATCGGTAGACGCTACG	1200 bp

2.4. Genomic DNA Isolation

The leaf pieces (200 mg) were collected in a sterile 1.5 mL microfuge tube. Plant tissues were ground into a fine powder, then added with 500 µL of 3% CTAB buffer. Plant extract mixture were incubated for 30 min at 60 °C in water bath. One volume of chloroform:isoamyl alcohol was

added, mixed well and incubated for 30 min in room temperature with shaking for homogenation, then centrifuged the mixture at 5000 rpm for 5 min. The upper phase solution was carefully transferred to a new sterile 1.5 mL tube. The volume of supernatant was determined. One tenth volume of Na acetate or cold absolute EtOH was added and mixed gently, then incubated at -20 °C for 10 min. Sample was centrifuged at 1000 rpm for 5 min and supernatant was discarded. The pellet was washed with 70% ethanol by centrifugation at 1000 rpm for 2 min. To dry up the DNA pellets, tubes with their lids open were inverted on sterile tissue paper for 30 min then DNA pellets were dissolved in 25-30 µL TE buffer/ Nuclease free water each. Isolated DNA samples were stored in -20 °C. The genomic DNA concentration in resulting solution (200 µL) was measured by spectrophotometer (Nanodrop 200C, ThermoScientific).

2.5. PCR test for transformant candidates

In this study the direct PCR method was performed using KOD Fx-Neo PCR Kit (Toyobo, Japan). The DNA template used was obtained by cutting a small protocorm (± 0.05 cm) of the plant transformant candidates using sterile tweezers embedded. Samples of plant parts were crushed by mixing 30 µL buffer solution (buffer solution A: 100

μM Tris-HCl pH 9.5; 1 M KCl; 10 mM EDTA) with micropestle. DNA templates were obtained from the supernatant. PCR was performed in a 25 μL volume containing 1 μL genomic DNA sample, 12.5 μL 2 \times PCR buffer for KOD Fx Neo, 5 μL 2 mM dNTPs, 0.5 μL KOD Fx Neo (Toyobo, Japan), 0.75 μL of each primers and 4.5 μL nuclease-free water. The PCR conditions were pre-denaturation (94 °C for 2 min), 30 cycles of denaturation (98 °C for 10 s), annealing (58 °C for 50 s), and extension (68 °C for 45 s). The PCR was performed using T100™ Thermal Cycler (Bio-Rad). The primer sequences are shown in Table 1.

2.6. Qualitative analysis of transformant

To find out whether there is a mutation at the target site in the transformant genome, a PCR with primers specific to the target gene was performed. Plant genome DNA were amplified by PCR using PDS primers and separated the PCR product using 4% low EEO agarose S (Nippon Gene, 313-90231). The low EEO agarose was dissolved in 1 \times Tris-Acetate EDTA (TAE) bufer (40 mM Tris-acetate, 0.5 mM EDTA pH 8.3, Fisher Bioreagents BP13324) by heating the solution in a microwave oven for 2–3 min. PCR with some primers: HPT, Cas9 and trnL-F were also conducted using specific primers and checked using 2% agarose gel (Sigma Aldrich type 2). The melted agarose gels were added with 5 μL of ethidium bromide (10 mg/mL), then it was immediately poured on a UV transparent gel casting tray. High concentration of agarose gels should be poured rapidly as the gel solidifies quickly. The electrophoresis chamber filled with 1 \times TAE buffer till about 1 cm above the gel. The total of 10 μL of each PCR sample was loaded into each well and electrophoresis was performed for 1 h and 40 min. Our power supply was set to 100 V. The 4% agarose gels were run at room temperature (25 °C). DNA size marker 1kb (Geneaid) is a ready-to-use solution containing 6 \times loading dyes. Gel images were acquired using a regular gel-documentation system (Bhattacharya and Van Meir 2019).

3. Results and Discussion

3.1. Plant genetic transformation mediated by *Agrobacterium tumefaciens*

In this research protocorms were used for transformation material using *Agrobacterium tumefaciens* strain EHA105 carried T-DNA with UBI::Cas9::U3::PDS in pRGE32. The transformation efficiency in *P. amabilis* using *PDS3T1* sgRNA is 0.9%, lower than transformation efficiency using *PDS3T2* sgRNA is 0.96% (Table 2). Transformation efficiency in this study were lower compared to the previous study. In *Dendrobium phalaenopsis*, transformation efficiency reached until 12% (Setiari et al. 2018) and *D. lasianthera* up to 70% (Utami et al. 2018). This might be caused by each species has their own genetic make up (Men et al. 2003). Moreover, transformation by using *Agrobacterium* was a biological approach that has various factor to get an optimum result, such as type explant, bacteria condition and pre-treatment (Chen et al. 2018).

Agrobacterium-mediated transformation is a method that enables production of stable transformants and as a model for studying the cellular localization and interactions between proteins that are important for the development of functional genomic analysis (Krenek et al. 2015). *Agrobacterium*-mediated transformation used by scientists can introduce new traits into plants through over-expression, knockdown or knockout the gene expression through *Agrobacterium*-mediated T-DNA transfer (Semiarti et al. 2007; Guo et al. 2019). *A. tumefaciens* uses a unique virulence mechanism to induce tumors, it delivers DNA fragments (transferred DNA or T-DNA) to host cells where the T-DNA ultimately integrates into the genome. The pathogenicity of *A. tumefaciens* is predominantly determined by a large Ti (tumor inducing) plasmid (Guo et al. 2019).

The phenotypic change was also detected from the green color reduction of almost all protocorms, that changes from green to black on the 4th week in the selection medium. The black phenotype of protocorm can be

TABLE 2 Efficiency of transformation in Protocorm *P. amabilis*.

Treatment	Repetition	Σ transformed protocorm	Σ survival protocorms in selection medium	PCR Detection			Efficiency of transformation (%)	Efficiency of Genome editing (%)
				HPT+	Cas9+	PDS+		
Non-transformant (Wild Type)	1	430	3	0/3	0/3	0/3	0	
	2	405	2	0/2	0/2	0/2	0	
	3	392	1	0/1	0/1	0/1	0	
	Total	1227	6	0/6	0/6	0/6	0	0
<i>PDS3T1</i>	1	420	4	1/7	1/7	1/7	0.95	
	2	385	3	1/3	1/3	1/3	0.77	
	3	295	3	1/4	1/4	1/4	1.0	
	Total	1100	10	3/14	3/14	3/14	0.90	0.19
<i>PDS3T2</i>	1	417	5	1/5	1/5	1/5	1.1	
	2	407	4	1/4	1/4	1/4	0.98	
	3	365	3	1/3	1/3	1/3	0.82	
	Total	1189	12	3/12	3/12	3/12	0.96	0.25

caused by hygromycin as a selection agent affected photosynthesis rate. As Mingeot-Leclercq et al. (1999), this antibiotic can bind 80 ribosomal complexes. Furthermore, the green protocorm was indicated as a transformant candidate plant (Figure 3).

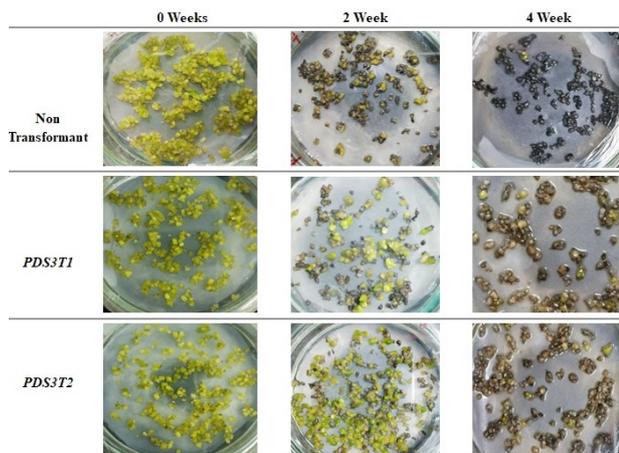


FIGURE 3 Development of *P. amabilis* protocorms on Hygromycin containing selection medium for transformants.

In this result the transformation efficiency was low (Table 2), it might be due to the low efficiency in T-DNA integration into the plant genome. Our previous study in *Dendrobium phalaenopsis*, efficiency transformation reached until 12% (Setiari et al. 2018) and Utami et al. (2018) got high transformation efficiency in *D. lasianthera* that the transformation efficiency up to 70%. This might be caused by specific character of each species related to their own genetic make up (Men et al. 2003). Moreover, transformation by using *Agrobacterium* was a biological approach that has various factor to get an optimum result, such as type of explant, bacteria condition and pre-treatment (Chen et al. 2018). *A. tumefaciens*-mediated genetic transformation has been routinely used in rice. The study used different cultivar rice with *A. tumefaciens*-mediated genetic transformation. However, the transformation efficiency of the indica rice variety is still much lower than that of japonica cultivars. This further improvement on the transformation efficiency lies in the genetic manipulation of the plant itself, which requires a better understanding of the underlying process in account for the susceptibility of plant cells to *Agrobacterium* infection as well as the identification of plant genes involved in the transformation process (Tie et al. 2012). Utami et al. (2018) used the five different infection period of *Agrobacterium* for another purpose. According to our result, futher research is necessary to increase mutation frequency for mutant propagation.

3.2. PCR-based detection of CRISPR/Cas9-edited in transformant plant

Transformant candidates were screened using PCR to confirm T-DNA integration. HPT and Cas9 primers were used to screen the candidates to confirm the transformations of pRGEB32 construct harboring T-DNA (UBI :: Cas9 :: U3

:: PDS). After that, 8 weeks-old green protocorms grown on selection medium were used as screening materials. We regenerated the transformant in NP media+ ZPT (NAA: BA 1:2). Protocorms were grown in regeneration media until they produced leaves for mutation detection according to Bhattacharya and Van Meir (2019) method.

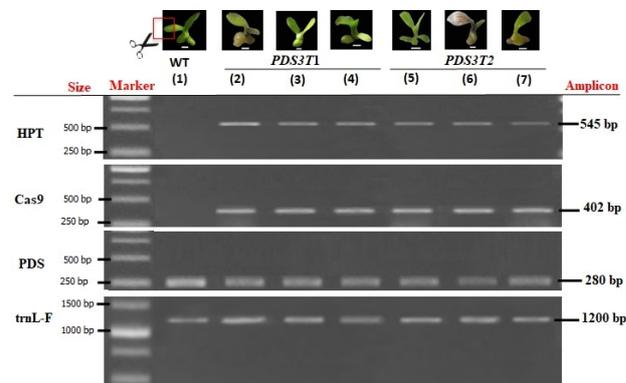


FIGURE 4 Detection of transformant orchid plants based on PCR-amplification of transgene. PCR were performed using specific primers of HPT, Cas9, and *PDS3* genes. The fragment of *trnL-F* was used as internal control of PCR reaction. (M: 1kb Gene Aid DNA Marker, 1: Wild type *P.amabilis*, 2-4: Transformant candidates *PDS3T1*, and 5-7: Transformant candidates *PDS3T2*).

The electrophoresis results (Figure 4) show that T-DNA has successfully entered the plant genome. This refers to the band that is detected appearing on some of the primers used. Results showed specific size of amplicon from each primer pairs, i.e HPT (504 bp), Cas9 (402 bp), PDS (280 bp) and *trnL-F* (1200 bp) as an internal control. Results showed no multiband or band shift on gel electrophoresis. This possibility does not occur in the genome insertion so that no extra thickening of the band or bands appear. So it needs to be confirmed by using Sanger Sequencing analysis method to see whether there are the mutations occurred or not.

3.3. Sequence analysis of CRISPR/Cas9-edited in transformant plant

Mutation detection in the genomes of transformant plants were analysed by using 4% agarose gel low EEO following the protocol of Bhattacharya and Van Meir (2019). Nevertheless, the drawback is that the amplicon size cannot be known accurately. It can only be estimated its size by comparing non-transformant with transformants and referring to the markers used. Further confirmation is required by sequencing and alignment to find specific changes in target sequence. In this research, we compared sequencing data from wild type/non transformant and transformant. We used *PDS3* gene with two target site namely *PDS3T1* and *PDS3T2*.

Sequencing analysis uses PCR amplicons produced from genomic DNA templates. Furthermore, the process of alignment of the transformant genomic DNA template that is identical to wild type sequences. This analysis showed that one of the edited protocorms kept a mutation (Figure 5) located on the CRISPR/Cas9 target site for

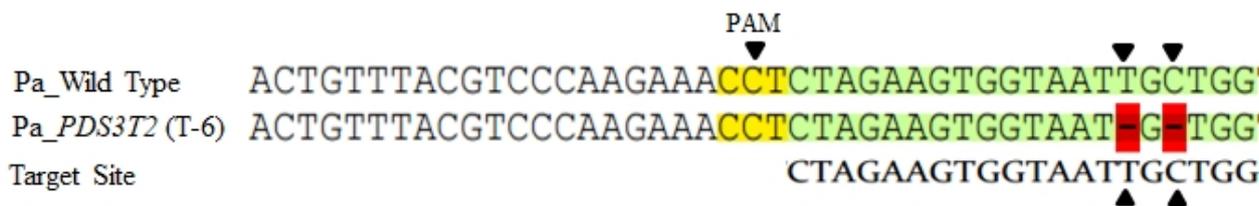


FIGURE 5 The sequence alignment of the edited transformant *PaPDS3T2* line against wild-type sequence produced in protocorm of *P. amabilis*. Highlighted nucleotide by red there indication has occurred mutation.

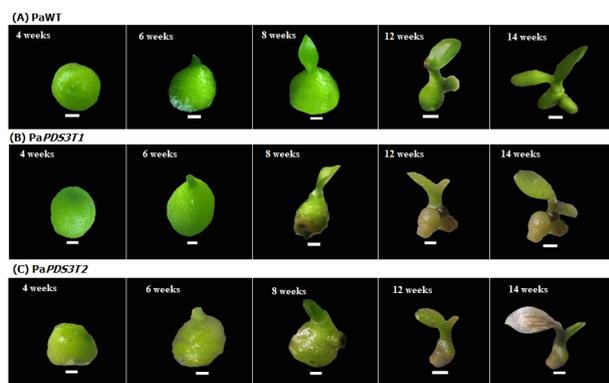


FIGURE 6 Phenotypic changes on the development of protocorm into shoot of *P. amabilis*. (A) Wild-type and the transformant (B) *PDS3T1*; (C) *PDS3T2*.

PDS3T2. This indicated that mutations occurred as a base deletions due to internal DNA repair, then the process of cutting double strand DNA and forming a double-strand break were caused by CRISPR/Cas9. The repair process is called NHEJ (Non-homologous end joining) gene mutation that results in a knock-out. This mutation induces changes in amino acid sequences which will also changes the phenotypic. Because of those carotenoid pathways disrupted causing an albino phenotype (Figure 6). In our previous study, to edit the homologous *PDS3* gene in *P. amabilis* orchids, we used the pKIR1.1 vector (Tsutsui and Higashiyama 2016) with the AtrPS5A promoter from *Arabidopsis*, but we did not get any mutants that showed insertion or deletion at the target site of *PDS3* gene. The pRGE32 vector that containing OsUbi-pro, a Ubiquitin promoter from rice (*Oryza sativa*) which is a monocot plant similar to an orchid, turns out that CRISPR/Cas9 can work properly in orchid, produced orchid mutant with color changes on the leaves because of the indel occurrences in their *PDS3* target sites.

Our in silico sequence alignment of the wild type and CRISPR/Cas9-edited *P. amabilis* T#6 *PDS3T2* (Figure 7) showed that deletion changed the translated amino acid sequence. Indicated by black triangles. Yellow highlight was PAM sequence. This data indicated that CRISPR/Cas9 can induced a mutation in the way of deletion. This phenomenon lead a frameshift on the translated amino acid sequence.

This shows that the CRISPR/Cas9 method can be used to edit orchid genome using appropriate vectors and pro-

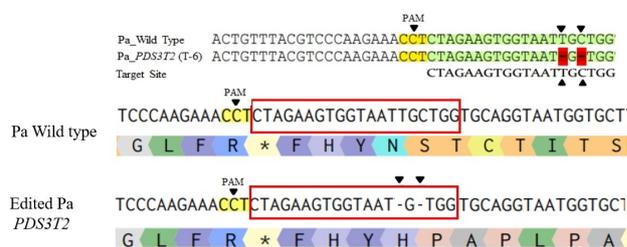


FIGURE 7 In silico Sequence alignment of Wildtype and edited *P. amabilis* T#6 *PDS3T2* in Benchling. Highlighted nucleotide by black triangles showed where the change made by CRISPR/Cas9 and changing the translated amino acid sequence.

moters.

4. Conclusions

CRISPR/Cas9 genome editing KO system can be applied to orchids, especially *Phalaenopsis amabilis*. The *PHYTOENE DESATURASE 3 (PDS3)* gene can be used as a model/marker gene which makes it easy to determine whether the two *PDS3* alleles are disturbed in the observed tissue. The *pds3* mutants of *P. amabilis* show albino phenotype in the leaf tissues. The use of CRISPR/Cas9 for *P. amabilis* is still in its early stages in the T0 generation, so further research is needed for the the next generation in order to obtain precise information about the effectiveness next generation of the editing genome in orchids, especially for KO suppressor functional genes in producing new trait.

Acknowledgments

This work was supported by Basic Research Grant from the Indonesian Ministry of Research, Technology and Higher Education with Contract No. 2791/UN1/DITLIT/DIT-LIT/LT/2019 given to E.S. as PI (2018-2019), and in part was supported by Japan Society for Promotion of Science (JSPS) Fiscal Year 2018-2019 with Contract No:219/D3/PG/2019 given to E.S. as PI.

Authors' contributions

ES, AP, JW, SM and YY designed the research. YY, KN and YA designed the sgRNA in plasmid pRGE32 cas-

settes. SN, YS and MDL conducted the genetic transformation bacteria and screening in antibiotic supplemented plates. SN conducted genetic transformation and YS conducted the isolation of genomic DNA, PCR. SN conducted, sequencing, subsequent analyzes, data compiling and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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