CRISPR/Cas9 system for disruption of biochemical pathway for sterol synthesis in *Artemisia annua* L.

S Koerniati[1]* and **G Simanjuntak**[2]

[1] Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, Jalan Tentara Pelajar No. 3A, Bogor 16111, West Java, Indonesia
[2] Department of Biochemistry, IPB University, Dramaga Campus, Jalan Meranti, Bogor 16680, West Java, Indonesia

*E-mail: srikoer818@gmail.com

Abstract. WHO recommends artemisinin-based combination therapy for curing malaria which is still a health problem in Indonesia. *Artemisia annua* L. is the primary source for artemisinin comes from subtropical China and India. Some introduction materials are grown in Indonesia, but they produce low concentration of artemisinin. Artemisinin synthesis uses the isoprenoid pathway, in which farnesyl diphospate (FDP) serves as the main precursor. FDP is the main precursor for sterol synthesis as well. The gene controls sterol biosynthesis is *Squalene synthase* (*SQS*). So then *SQS* is considered a competitive gene for artemisinin biosynthesis. CRISPR/cas9 is the most advanced system for genome editing, and can be used to induce a targeted-mutation. The aim of this research was to elevate Artemisinin content through disruption of the biochemical pathway of sterol synthesis using CRISPR/Cas9. Materials and methods used were as follows. *Artemisia* CRISPR/Cas9 seeds were processed and grown in selection medium and then soil. Seventy-seven *Artemisia* kanamycin resistant lines were produced. Fifty-two (of 77) lines were confirmed to have T-DNA by PCR with SQS-Fn and SQS-Rn primers, produced about 710 bp DNA fragment. By using SQS-Fs and SQS-Rs primers, those produced shorter DNA fragments (about 470 bp). A high-resolution electrophoresis QIAxcel was applied to identify for any nucleotide difference occured in PCR product of lines. Results showed that 44 lines (84.61%) have varied sizes with one to three nucleotides differences compared to control (plasmid, 468 bp). By assuming this analysis was right, it may be said that mutations has occured in *SQS* gene of *Artemisia* lines due to the CRISPR/Cas9.

Keywords: *Artemisia annua* L., artemisin, *squalene synthase* (*SQS*) gene, CRISPR/Cas9, targeted-mutation.

1. Introduction

As a world health problem, Malaria caused by *Plasmodium palciparum* has been treated by so many cures found for centuries. For a long time, quinine has been used for treating malaria, but in the 1960s *P. palciparum* started to show the signs of resistance against quinine-derived drugs [1]. In 2001, The World Health Organization (WHO) recommends Artemisinin Based Combination Therapies (ACTs) as the most effective way to treat malaria [2]. To date, *A. annua* is still the main commercial source of

artemisinin. Besides its antimalarial function, artemisinin has also been reported to have antiviral, anticancer and antischistosomal, resulting in its high demand by the pharmaceutical industry. Unfortunately, the supply of artemisinin is significantly restricted by the low content of artemisinin (0.01–0.1% leaf DW) in *A. annua* [3]. A promising approach to enhance the content of artemisinin and consequently to reduce the price of artemisinin is to use plant metabolic engineering to obtain a higher content of artemisinin in transgenic plants. It is possible now to regulate the biosynthesis of artemisinin in a direct way because several genes that are critical for synthesizing artemisinin have been cloned [3]. The synthesis of artemisinin belongs to isoprenoid pathway. In this pathway, farnesyl diphospate (FDP) serves as the main precursor for synthesis both sterol and sesquiterpen, such as artemisinin. The gene control biosynthesis of sterol is *Squalene synthase* (*SQS*) while artemisinin controlled by amorhpha-4, 11-diene synthase (ADS). *SQS* is considered as a competitive enzyme for artemisinin biosynthesis [4] (Figure 1).

Therefore, to increase Artemisinin content of *Artemisia*, the expression of *SQS* gene must be inhibited. In order to inactivate this gene, a clustered regularly interspaced palindromic repeats/ CRISPR-associated protein Cas9 (CRISPR/Cas9) which is the most advanced system for genome editing can be used to induce a targeted-mutation. CRISPR/Cas9 is revolutionizing genome editing technology with minimal off-targets in the present era. Genome editing using CRISPR/Cas9 utilizes a 20-bp guide RNA (gRNA) sequences that uses base pairing to direct Cas9 nuclease to target site and generates double-strand breaks (DSB). Following DSB is DNA repairing mechanism. During DNA repairing process, mutations are often introduced [5]. Besides gene deletions, CRISPR/Cas9 is useful for inserting specific DNA fragment into target sites and specifically altering the transcriptional activity of genes by fusing transcriptional activation or repression domains to an inactivated Cas9 [6,7]. The objective of this research was to elevate artemisinin concentration through disruption of the biochemical pathway of sterol synthesis using the CRISPR/Cas9 system. This is the first report about attempts to apply CRISPR/Cas9 system in *Artemisia*. We produced CRISPR/Cas9 vectors to targeting of *SQS* gene and about 44 *Artemisia* CRISPR/Cas9 lines indicated for some mutations.

2. Materials and methods

2.1. Seeds preparation

Seeds were harvested from 10 individual stalks each of four plants. One plant was either transformed with *Agrobacterium tumefaciens* containing of pHEE401 or pKSE401 plasmid. Both plasmids were transformed by *A. tumefasiens* strain AGL1 and GV3101. Seeds were sterilized using 10% of sodium hypochlorite, 70% of ethanol and 1% of plant tissue culture (PSM). Sterilized seeds were cultured in Murashige and Skoog (MS) medium with additional about 50 mg/l hygromycin (antibiotic) as selectable marker. While for seeds from plants which were transformed with pKSE40, 50 mg/l kanamycin was applied. Seeds of the wild type of *Artemisia* were grown on similar media without antibiotic. All samples were placed in the cool growth room (24–28°C) for seeds germination.

2.2. Acclimatization

Seedling of *Artemisia* were transplanted into the soil medium in polybags (diameter of 30 cm) and maintained in the plastic house at Balithi, Cipanas. Plants were grown in high land altitude about 1,000 m above sea level which is an optimum condition for *A. annua* to grow appropriately. Three months after planting, observation on plant morphology was conducted. Observation was included the shape of leave and stem, plant height and number of branches.

2.3. DNA preparation

DNA was isolated from young fresh leaves using a CTAB method (according to Agrawal et al. [8]) with some modification. Leaves were crashed in 2 ml eppendorf tube using chop-stick and with addition of liquid nitrogen. Extracted DNA was dissolved with 50 µl TE buffer and 1 µl RNase (10 mg/ml) was added to remove RNA. After that, the DNA solution was checked quantitatively using

Nanodrop spectrophotometer and qualitatively using 1% agarose gel electrophoresis and visualized using Chemidoc.

2.4. PCR for detection

DNA that diluted with nucleus free water (NFW) into 10 ng/µl of DNA was used as a template for PCR. The total reaction of PCR 10 µl consisted of 1.9 µl NFW, 5.0 µl 2× 2G Ready Mix KAPA, 0.6 µl $MgCl_2$ 25 mM, 0.5 µl DMSO, each 0.5 µl forward and reverse primers (10 nM/µl) and 1 µl DNA (10 ng/µl). Two pair of primers was used for identification of *Artemisia* CRISPR/Cas9 lines (Table 1). PCR products were checked by electrophoresis and by high resolution electrophoresis QIAxel (Qiagen) in order to obtain the approximate size of DNA amplicon of samples.

Table 1. Two pair of primer used for identification of *Artemisia* CRISPR/Cas9 lines.

Primers	Sequences
AaSQS-Fn	5'-TTTCTGAAGGTGGTACAAAGG-3'
AaSQS-Rn	5'-TTCGCCTGAAGAATGGAAGAG-3'
AaSQS-Fs	5'-GTGGTACAAAGGAATACAAAGTTCTC-3'
AaSQS-Rs	5'-CTCTTTACATATAAATTTTGCCATCC-3'

3. Results and discussion

3.1. Seed preparation and planting

Since two CRISPR/Cas9 plasmid vectors were inserted into the plant by two strains of *Agrobacterium*, GV3101 (coded by G) and AGL1 (coded by A), then we have four populations. They were labelled as G0 and A0 when containing of pHEE401 (with hygromicin resistant gene, *hpt*II) and transformed by Agrobacterium GV3101 and AGL1, respectively. Such similar populations, plants were labelled G2 and A2 since they contained pKSE401 (with kanamycin resistant gene selectable marker, *nptII*). Sterilized seeds for each seed batch were screened or selected using either hygromicin (50 mg/l) or kanamycin (50 mg/l) antibiotic in MS medium. During selection processes, several populations showed growth (Figure 1A). The surviving plants were considered as a transformed plant (transformant). It may indicate that the plasmid has been integrated into the plant genome. Nevertheless, some dying plants were found in medium MS and it may inhibit the transformed plants by secreting inhibitors or preventing transport of essential nutrients [9]. Then, the transformed plants were moved to new MS medium (Figure 1B). When plants grow bigger, they were immediately transplanted into the soil medium within polybag in the glass house for acclimatization (Figure 1C).

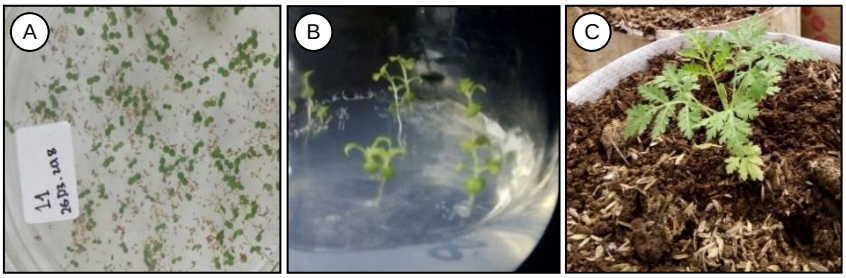

Figure 1. Preparation of *Artemisia* CRISPR/Cas9 plant. (A) Seeds selection in MS medium containing of antibiotic. (B) Transformant plants in new MS medium containing of antibiotic. (C) Tranformants plant transplanted into soil in polybag (diameter 30 cm).

3.2. Acclimatization

Until the acclimatization period finished, there were 77 survival plants/lines consisting of 7 lines of A0, 29 of G0, 40 plants/lines of G2, and 1 non-transgenic plant. So it is assumed that 36 lines contained T-DNA of pHEE401 and 40 lines contained T-DNA of pSKE401. Plants phenotype seemed slightly various and some variation in leaf arrangement are shown in Figure 2 (A, B and C). The main color of the stem and leaf are dark green but there are a few light-green leaves. The height of the plants reached 2.5 meters. Five of 77 plants (6.5%) have more than one main axis (symphodial) at the bottom part of plants, but most of the plants are monopodial. Since *A. annua* has been spreaded around the countries, it is possible that the phenotype of *A. annua* in each country is different. The diversity of phenotype may affect the content of artemisinin.

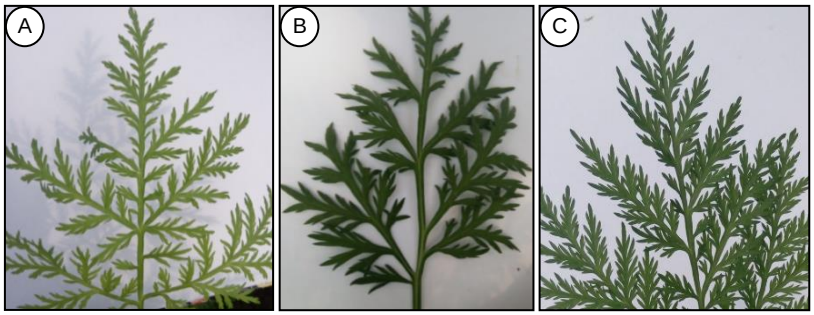

Figure 2. The representative of variation of *A. annua* leaves. (A) Distant-light green. (B) Distant-dark green. (C) Compact-dark green.

3.3. DNA Preparation

DNA isolation method for *A. annua* was a critical point for its result and will influence the molecular analysis result as well. Using liquid nitrogen, CTAB buffer and purifying agent successfully generated good extracted DNA. The quantity of extracted DNA was checked by spectrophotometer, and DNA concentration ranged from 78.8 up to 6,225.2 ng/µl. Both the quality and the quantity of extracted DNA were appropriate for molecular analysis.

3.4. PCR Detection

Based on the electrophoresis of amplified DNA (PCR product), results showed (Figure 4A, 4B and 4C) that 52 lines produced about 700 bp of DNA fragment. These mean T-DNA of CRISPR/Cas9 plasmid had been inserted into the *Artemisia* plant genome. In contrast, 25 lines did not produce such amplification of 700 bp DNA fragment (about 32.47%) and this should be investigated further, since plants were germinated and grown on MS selection medium after sterilization.

It has been reported that Cas9 protein endonuclease will do DNA double strands cutting at 3 to 4 bp after the protospacer adjusting motif (PAM) site, directed by guideRNA and DNA repair process often makes mistakes. It gives possibilities for deletion and or insertion to occur. Based on those, gRNA that was designed using E-CRISP based upon SQS cDNA gene sequence (GenBank: AF405310.1), after found complement in the genome will direct Cas9 protein endonuclease to cut the DNA with precision. Then it is expected to induce some mutation [5].

Conventional electrophoresis equipment was not able to show such differences (in size of DNA fragment produced by PCR) (Figure 3A, 3B and 3C) induced by mutation. Because of that, we tried to use a high-resolution electrophoresis (HRE) QIAxcel (Qiagen) to analysis PCR product of *Artemisia* CRISPR/Cas9 lines before to do sequencing. The equipment was designed to analyses DNA fragment with accuracy down to 3 to 5 bp (protocol). It, however, is only possible applied for DNA with size of smaller than 500 bp. Another set of PCR was made using another pair of primer (SQS-Fs and SQS-Rs) (Figure 4A). By using this equipment, amplicons can be measured and visualized as shown in Figure

4B. For example, sample number 12 has a size of 465 bp (Figure 5C). From these analyses, we managed to get data that showed differences down to 1 to 3 nucleotides compared to the plasmid.

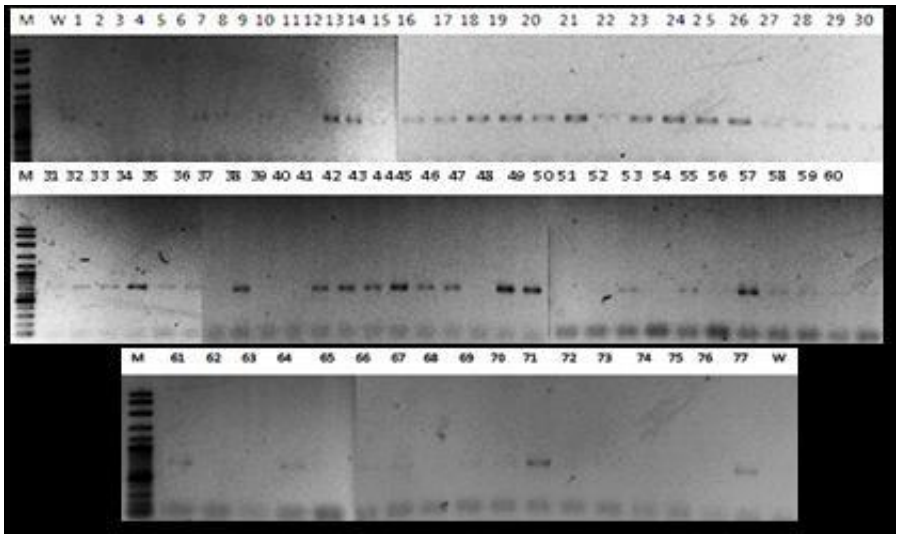

Figure 3. Electrophoregram of PCR product produced by *Artemisia* CRISPR/Cas9 plants. A = samples 1–30, B = samples 31–60, C = samples 61–77.

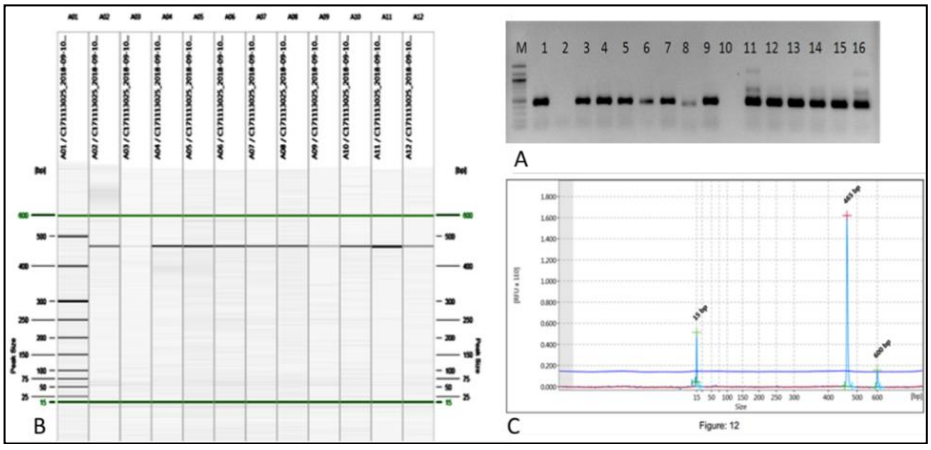

Figure 4. Electrophoregram of PCR product of *Artemisia* CRISPR/Cas9 using two types of electrophoresis. (A) Conventional. (B) High resolution electrophoresis (QIAxcel). (C) Graphyc shows the size of PCR product of line number 12.

From PCR product analysis using HRE QIAxcel, we obtained 44 of 52 *Artemisia* CRISPR/Cas9 lines had changes in their size compared to fragment resulted by the plasmid. Changes encountered were 84.62% (average) has small number deletion between 1 to 3 nucleotides (Figure 5). These may indicate that about 84.62% of lines were mutated. While another 15.38% of lines were not mutated, because they have similar size of DNA amplification (amplicon) as the control plasmid (468 bp). Of that 84.62% of mutated lines, about 69.23% had 1 nucleotide deletion, 11.54% had 2 nucleotides deletion and only about 3.83% of lines had 3 nucleotides deletion.

These experiments results may indicate that designed gRNA for *SQS* gene (target) can direct Cas9 protein endonuclease to do DNA breaks. For sizes confirmation, sequencing of PCR products are

needed. Then the next step is to align sequences of CRISPR/Cas9 lines compared to the *SQS* coding sequence (Genebank: AF405310.1) thath may lead to conclusion whether any protein changes and or an early stop codon has occured. Concentration of artemisinin of these 52 lines is being analyses. Both sequence and artemisinin information data may be sought for its corellation.

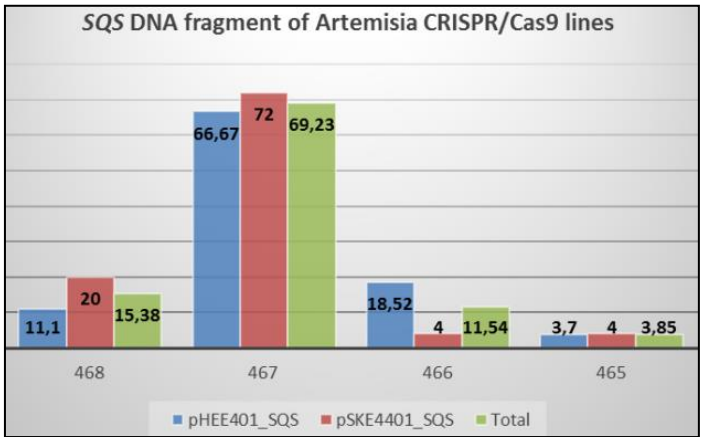

Figure 5. Percentages of CRISPR/Cas9 lines with nucleotide reductions (1 up to 3) induced by pHEE_SQS, pSKE401_SQS plasmids.

4. Conclusions

This is the first report about application of the CRISPR/Cas9 system onto *Artemisia*. Experiments carried out were successfull in generating of 52 *Artemisia* CRISPR/Cas9 lines and induced nucleotides changes in size of *SQS* gene of 44 *Artemisia* CRISPR/Cas9 lines (84.67%). Analysis of PCR products using high-resolution electrophoresis QIAxcel abled to show nucleotide differences among PCR products of lines, varied from 1 to 3 nucleotides. DNA sequencing and artemisinin content analysis are being carried out for confirmation. These would be able to find out whether nucleotide deletions affect the DNA sequence shifted, consequently changed the protein or *SQS* gene transcriptional activity. Information data on artemisinin concentration of these 44 lines will give a further confirmation about mutation that induced by CRISPR/Cas9 system, which can disrupt sterol synthesis and consequently increased the artemisinin content.

5. Acknowledgement

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