January 2022

THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture

Committees: The Second International Conference on Genetic Resources and Biotechnology

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Preface: The Second International Conference on Genetic Resources and Biotechnology

The Second International Conference on Genetic Resources and Biotechnology, which is the continuation of the first event held in 2018, focuses on topics related to advances in biotechnology to create more opportunities for effective conservation and sustainable utilization of genetic resources for food and agriculture. This year conference's theme is Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture. The conference was organized by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Indonesia, in collaboration with Indonesian Biotechnology Consortium and held on 24th-25th of May 2021virtually due to the pandemic of COVID-19.

The conference aims to share and exchange current scientific information and technological developments on biotechnology and their applications for conservation and sustainable use of genetic, to encourage and promote quality, efficiency, and modernization of management and utilization of genetic resources, and to facilitate national and international collaboration among participants. There are five scopes discussed in this conference. They are effective management of conservation and sustainable use of genetic resources for food and agriculture, application of genomics and molecular markers for genetic resource conservation and crop adaptation to climate change, application of innovative crop improvement techniques for conservation and sustainable use of plant genetic resources for food and agriculture, plant cell and tissue culture for conservation and effective utilization of genetic resources, and the use of microbial genetic resources as biological control agents of agricultural pests and diseases, and for soil bioremediation.

Five speakers from the United States of America, Japan, India and Indonesia were invited to discuss about their expertise and knowledge on relevant subjects in the plenary sessions. This conference was attended by more than 100 participants including 75 presenters and 44 listeners worldwide. They came from diverse governmental, private, or academic institutions and also scientific communities. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double blind peer-review and expected to meet the scientific criteria of significance and academic excellence to be published in a conference proceedings indexed in a well-known, reputable service.

We would like to express our sincere gratitude to our speakers, presenters and all participants for their contributions in this conference. We would also like to express our appreciation for the generosity of our sponsors that support this conference: PT CropLife, PT ITS Science Indonesia, PT Fajar Mas Murni and PT Prima Instrument Analitika. Lastly, special thanks to all committee members for their exceptional work and contributions in the conference and publication.

Chair of Organizing Committee

Dr. Toto Hadiarto

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Design and *in vitro* test of sgRNA for the CRISPR/Cas9 plasmid construct of the *SQS* gene of *Artemisia annua* L.

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Sri Koerniati

Design and *In Vitro* Test of sgRNA for the CRISPR/Cas9 Plasmid Construct of the *SQS* Gene of *Artemisia annua* L.

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Abstract. Artemisinin is the main compound of the dihydroartemisinic acid (DHA)-based antimalarial drug. Its synthesis might be impeded by *squalene synthase* (*SQS*) gene. This study aimed to design and synthesis sgRNA of *SQS* gene of *Artemisia annua* L., to amplify DNA fragment of the target gene, and to set up in vitro test to finding out sgRNA function. The CRISPR/Cas9 system is applied as it provides a more precise modification of the gene sequences. This preliminary study started with designing single guide RNA (sgRNA) and investigating their functions in directing the Cas9 protein to cut the target. The methods included designing sgRNA and primers using available tools online, synthesizing sgRNA, amplifying SQS DNA target, and setting up *in vitro* testing using kits and according to manufacturer instructions. The experiment resulted in two sgRNAs and two *SQS* DNA fragments (targets) that were incubated with the Cas9 protein at 37°C for 3 h in the *in vitro* test reaction. Both sgRNAs functioned since both 1.6 and 1.4 kbp DNA fragments became two smaller DNA fragments. These indicate that the CRISPR/Cas9 vector construct to target the *SQS* gene in the *A. annua* plant can use these two sgRNAs.

INTRODUCTION

Malaria caused by *Plasmodium falciparum* sickens and kills people. Globally malaria caused mortality of about 782 thousand in 2000 and still caused mortality of about 460 thousand in 2019 [1]. Many countries, such as Nigeria, Congo, and Tanzania contributed the highest, the 2nd, and the 3rd to the mortality of about 23, 11, and 5%, respectively. According to the World Health Organization (WHO) report, Indonesia contributed to 1% of malaria mortality which was reduced from 432 in 2010 to only 49 in 2019 [1].

For more than 50 years, chloroquine was used as an antimalarial drug, but between 1960 to 1970 its effectiveness against *P. falciparum* in Southeast Asia is lost, and then failed to treat malaria in Africa [2] as well. Among antimalarial drug replacements, artemisinin derivatives offer the broadest antimalarial action against the range of developmental stages of plasmodium and the fastest *in vivo* activity [3]. In addition, artemisinin derivatives are the only first-line malaria treatment to early-stage (gametocyte), prevents parasites (the ring) from maturing to the more pathological stages. Whereas quinine and quinidine only affect parasites when they are already in the later developmental stage (cytoadherence) which are primarily responsible for life-threatening complications [4]. In 2001, the WHO recommended the use of artemisinin-based combination therapy for effective treatment against malaria [1].

Up to now, *Artemisia annua* L. is still the main source of artemisinin. However, the artemisinin supply is significantly restricted by the low content of artemisinin (0.01 up to 0.1%) in *A. annua*. The synthesized artemisinin belongs to the isoprenoid pathway in which farnesyl diphosphate (FDP) serves as the main precursor for synthesis sesquiterpenes, the artemisinin, and synthesis of a sterol. There is a competition in using FDP for both biosynthesis in the *A. annua* plant. The sterol biosynthesis is controlled by a *squalene synthase* (*SQS*), whereas artemisinin is controlled by an *amorpha-4,11-diene synthase* (*ADS*). The *SQS* is therefore considered as a competitor gene in using of precursor for artemisinin biosynthesis (Fig. 1) [3].

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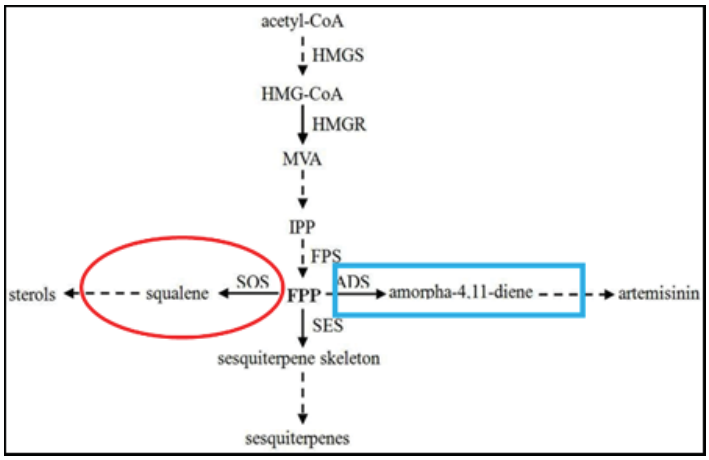


FIGURE 1. Biosynthesis pathway of artemisinin and sterol in *Artemisia annua* L. plant [3].

An effort to inactivating this *SQS* gene might increase the artemisinin within the *A. annua* plant. Several genetic engineering technologies have been developed to inactivate gene targets, e.g. zinc-finger nucleases (ZFNs), transcription activator-like effector nuclease (TALEN), and an RNA-guided nucleases system, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). CRISPR is the most advanced technology for inactivating gene target, consisting of a guide-RNA (gRNA) of gene target and Cas9, a DNA nuclease protein. This system was developed from a defense mechanism of bacteria and archaea to silence foreign nucleic acids of viruses and plasmids [4]. These defense systems rely on small RNAs for sequence-specific detection and silencing of foreign nucleic acids [4]. The CRISPR/Cas9 gives more benefits than ZFSs and TALENs, e.g. simplicity of customization, higher targeting efficiency, and the ability to facilitate multiplex genome editing [5]. This Cas9 protein binds to sequences directed by the gRNA, and cuts double-stranded DNA (dsDNA) of the gene target, at adjacent of the protospacer adjacent motif (PAM) [6]. In many plant species, its application for mutagenesis is now a routine procedure, if suitable target sites, sufficient expression of the Cas9 protein, and functioning single guide RNA (sgRNA) are combined [7]. This study aimed to design and synthesis sgRNA of *SQS* gene of *A. annua*, to amplify DNA fragment of the target gene, and to set up *in vitro* test to finding out sgRNA function.

MATERIALS AND METHODS

Designing of Single Guide RNA

There were two steps to design a sgRNA. The first step was to find sequences of the target gene from GenBank (NCBI) and the second one was used to find the sequences of the target gene. sgRNA and PAM sequences were examined using the online tool E-CRISP Design (http://www.e-crisp.org/E-CRISP/). By using the E-CRISP online tool, all possibilities sgRNA was searched. The non-specificity of the target sequence was also checked. The position of sgRNAs in the gene's open reading frame (ORF) was checked and confirmed using Vector NTI Advance 11 program (Invitrogen).

Single Guide RNA Synthesis

sgRNA was synthesized using the EnGen sgRNA Synthesis Kit, *Streptococcus pyogenes* (New England Biolab/NEB) according to the manufacturer's protocol. In doing so, an artificial sgRNA (oligos), coded by ArtsgRNA, was needed. NEB provided the EnGen sgRNA template for designing oligos that can be accessed online (https://sgrna.neb.com/#!/sgrna). Oligos sequence was composed of the T7 promoter, target DNA (sgRNA), and RNA scaffold. These were ordered from Integrated DNA Technologies (IDT). Reaction mixture of 20 µl consisted of 5 µl target-specific DNA (ArtsgRNA), 10 µl 2× EnGen sgRNA buffer, 1 µl EnGen sgRNA enzyme, and 4 µl nuclease-free water (or DEPC water) was incubated at 37°C for 30 min. Then, the reaction was brought into ice and brought the volume up to 50 µl by adding DEPC water and treated by DNaseI (2 µl) and kept in ice for 15 min.

Lastly, the reaction proceeded with the RNA purification step. The purification was carried out using TRIzol-based RNA purification kits (Zymo Research), according to the manufacturer's protocol. A concentration of purified RNA was checked by NanoDrop ND1000 (Thermo Fisher Scientific) and by agarose gel electrophoresis.

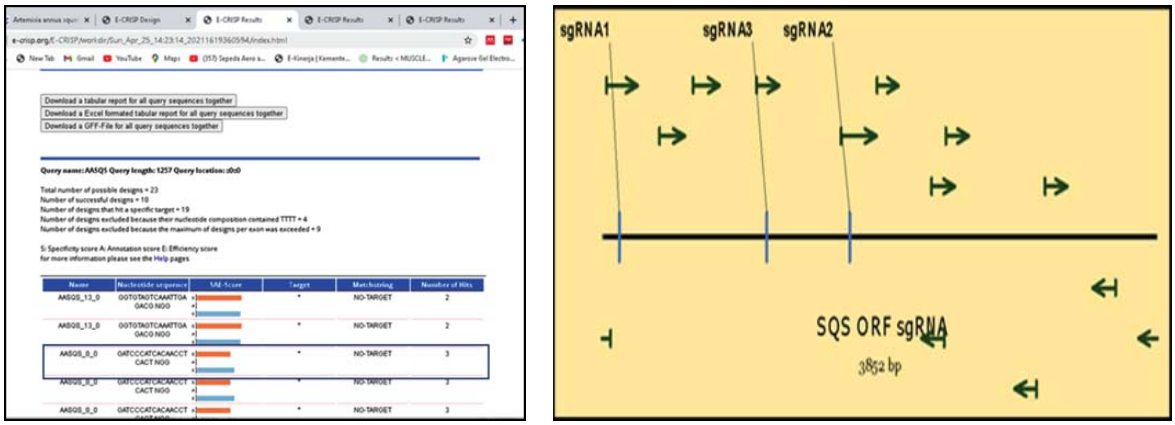
In Vitro Testing of Single Guide RNA

A DNA target for the *in vitro* testing was prepared by PCR, using MyTaq DNA polymerase (Bioline). Primers that were designed by Primer 3Plus program were used to amplify DNA target from genomic DNA of *A. annua*. Reaction was set up according to the manufacturer's instructions (Bioline). *In vitro* test to find out whether designed sgRNAs were able to recognize the target and direct the Cas9 protein to do cutting or inducing double-strand break (DSB) of DNA, was conducted by mixing of sgRNA, Cas9 protein, and substrate (target DNA) in 10:10:1 molarity ratio (NEB protocol). The Cas9 nuclease of *S. pyogenes* was applied in this *in vitro* test reaction, according to the manufacturer's instructions. In 15 µl of reaction, it consisted of 1.5 µl 10× Cas9 buffer, 12.5 µl of sgRNA (concentration of 40 ng/µl), 0.5 µl of Cas9 nuclease (concentration of 1 µM), and 0.5 µl of the substrate (concentration of 100 nM/µl). The substrate was added into the reaction after the first three components were incubated at 25°C for 10 min, then the reaction was incubated at 37°C for 3 h (NEB protocol). The reaction was checked by 1.2% agarose gel electrophoresis and visualized using GelDoc 2000 and UV transilluminator.

RESULTS AND DISCUSSIONS

Designing of Single Guide RNA

DNA of the *SQS* gene resulted in a complete cDNA of the *SQS* gene, coded by AF405310.1, as a reference gene (https://www.ncbi.nlm.nih.gov/nuccore/AF405310.1/). The gene size is about 3,852 bp consisting of 13 exons (direct DNA cleavage by Cas9 to the target sequence) [4]. The type II system, Cas9 protein constitute a family of enzymes that cleave target dsDNA via a base pair structure generated between activation tracrRNA and the targeting crRNA. The presence of PAM at the target DNA region determines how the nuclease targeting to a particular locus [8]. A PAM at the 3' end adjacent to the 20-base-pair target site is required, and a different Cas9 uses different PAM for the target site [9]. The PAM sequence of NGG is the most effective trinucleotide for the *S. pyogenes* CRISPR-Cas9 system [10].



(a) (b)

FIGURE 2. Single guide RNA (sgRNA) for targeting the *SQS* gene of *Artemisia annua* L. (a) Design of sgRNA of *SQS* gene resulted from E-CRISP online tool (http://www.e-crisp.org/E-CRISP/). (b) Position of sgRNAs in the *SQS* gene sequence and ORFs map made by Vector NTI Advance 11 program.

We designed sgRNA for targeting the *SQS* gene by putting the *SQS* cDNA sequence into the E-CRISP online tool (Fig. 2a). This tool was developed by academic labs and supporting gRNA design for several species [8]. This activity resulted in three sgRNAs, i.e. sgRNA1 (GATCCCATCACAACCTCACT) (signed by a box, Fig. 2a),

sgRNA2 (GAGTTTTGTCATTATGTTGC), and sgRNA3 (GCCTTTCTGGAACTTAAGAG), and each sgRNA has trinucleotide of GGG, GGG, and AGG, respectively. We located these sgRNAs in ORFs of the *SQS* gene sequence map using the Vector NTI Advance 11 program (Fig. 2b). This step is essential in designing the CRISPR construct for knockout via non-homology end joining (NHEJ) because it targets a common exon [8].

Single Guide RNA Synthesis

After sgRNA was designed, the next step was to synthesize these sgRNAs using a kit available for *in vitro* test. We utilized the EnGen sgRNA Synthesis Kit, *S. pyogenes* (from NEB), to synthesize sgRNAs. One must define in designing of sgRNA for *in vitro* test is the promoter to express the guide RNA [8]. This NEB system has the T7 promoter to drive sgRNA, so each oligo had a T7 promoter in front of the sgRNA sequence and a part of the RNA scaffold. This oligo design facilitates the Cas9 protein to bind to the target, *SQS* gene of *A. annua* (Fig. 3).

(a)

```
ArtsgRNA1: TTCTAATACGACTCACTATA GATCCCATCACAACCTCACTGTTTTAGAGCTAGA
ArtsgRNA2: TTCTAATACGACTCACTATA GAGTTTTGTCATTATGTTGCGTTTTAGAGCTAGA
ArtsgRNA3: TTCTAATACGACTCACTATA GCCTTTCTGGAACTTAAGAGGTTTTAGAGCTAGA
```

(b)

```
GAGUUUUGUCAUUAUGUUGCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAU
CAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
```

FIGURE 3. Oligo design to facilitate the Cas9 protein to bind to the *SQS* gene of *Artemisia annua* L. (a) Target-specific DNA oligo template (5′→3′). (b) The sgRNA3 sequence transcribed. Red: T7 promoter, green: sgRNA, gray: overlap/RNA scaffold.

This experiment step resulted in RNA. For example, sgRNA3 yielded about 320.7 ng/µl after the purification (Fig. 4, lane N). This RNA was aliquot in 40 ng/µl working stock (Fig. 4, lane W) for *in vitro* testing. These RNAs were kept at -80°C for further use.

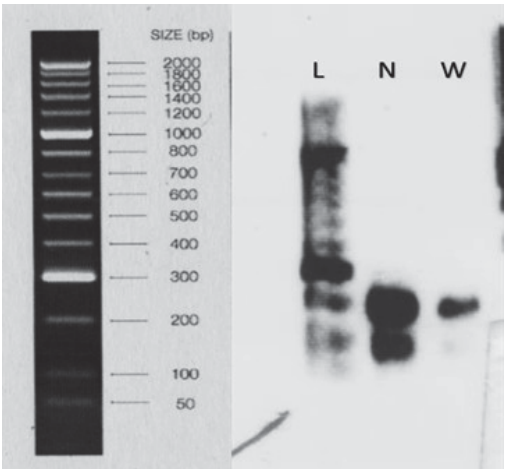
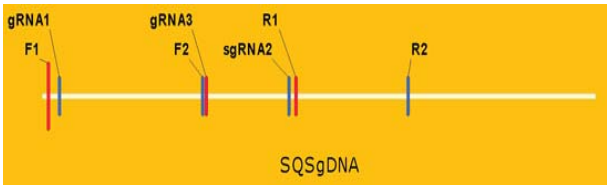


FIGURE 4. Electrophoregram of purified RNA (sgRNA3) resulted from the synthesis. L = 50 bp ladder, N = isolated RNA (320 ng/µl), W = working stock of RNA (sgRNA3; 40 ng/1µl).

In Vitro Testing of Single Guide RNA

The next step before we proceed to the *in vitro* test for sgRNA was to prepare the *SQS* gene fragment as a target for Cas9 protein cleavage by PCR. The position of primers used for amplification of DNA fragment (target) in the *SQS* gene map and their sequences are shown in Fig. 5.



(a)

1	ArtDNAF1	5'-AGCAGTATTGAAACACCCAGATG-3'
2	ArtDNAR1	5'-CCGTGCCTGAAGAATGGAAG-3'
3	ArtDNAF2	5'-GGACCAGTTCCATGTTTC-3'
4	ArtDNAR2	5'-ACACCCCTGAAAACCTCAATGT-3'

(b)

FIGURE 5. Position of primers and sgRNAs in the *SQS* gene sequence map made by Vector NTI Advance 11 (a) and primers sequences used to amplify DNA target (b).

PCR products were about 1.6 kbp and 1.4 kbp of DNA fragments (targets). The purified DNA fragments were checked using agarose gel electrophoresis (Fig. 6) and measured the concentration using NanoDrop. These DNA fragments become a target for the Cas9 nuclease protein to cut directed by the sgRNA provided in the *in vitro* reaction.

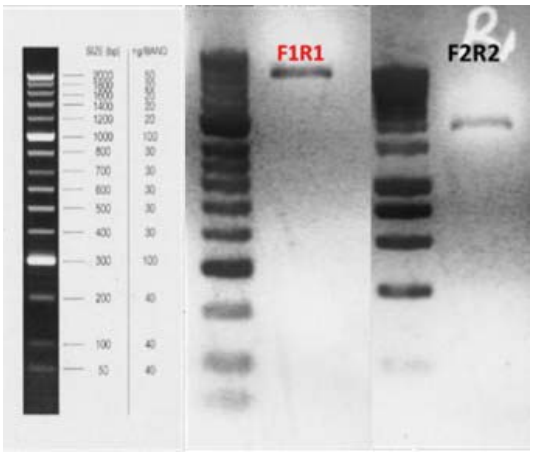


FIGURE 6. Amplicon resulted from PCR. F1R1 = using F1/R1 primer pair, F2R2 = F2/R2 primer pair.

The *in vitro* reaction with the Cas9 protein and sgRNA showed that both DNA fragments were cut into smaller DNA fragments after incubation for 3 h (Fig. 7). The target DNA of about 1.6 kbp long was cut into approximately 0.9 kbp and 0.7 kbp of DNA fragments, whereas the other one was cut into 0.9 kbp and 0.5 kbp of DNA fragments. These results indicate that both sgRNA2 and sgRNA3 direct the Cas9 protein to the sites and do DNA cutting. These *in vitro* results related to what was mentioned by Bente *et al.* [7]. When the Cas9 protein and sgRNA are co-expressed, they form a functional ribonucleotide protein (RNP) complex via the scaffold sequence of the sgRNA and target the genomic site by the specific and complementary part of the sgRNA [7].

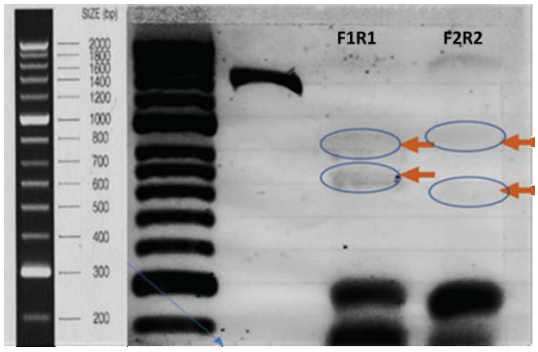


FIGURE 7. Electropherogram of DNA fragments resulted from *in vitro* test. 1 = DNA target (1.6 kbp), 2 = two DNA fragments (0.9 kbp and 0.7 kbp) of DNA target F1R1, 3 = two DNA fragments (0.9 kbp and 0.5 kbp) of DNA target F2R2.

The Cas9 is the most widely applied endonuclease, which more reliable partner in the CRISPR system. This nuclease makes the sgRNAs the main determinants of success or failure of targeted mutagenesis [11]. An assessment of sgRNA efficiency, especially mutagenesis with expensive transgenesis, is often obtained by *in vitro* assembly of candidate sgRNA with recombinant Cas9 protein and test the nuclease activity on the target DNA [7].

CONCLUSION

sgRNA2 (GAGTTTTGTCATTATGTTGC) and sgRNA3 (GCCTTTCTGGAACTTAAGAG) that developed from this experiment successfully directed the Cas9 protein to recognize, binding, and inducing DSB of DNA target. Thus, these sgRNAs can be used in the CRISPR construct to induce mutation at the *SQS* gene of *A. annua*. *In vitro* test is a useful step before a sgRNA can be used in a CRISPR construct. *In vitro* test can also be used as a protocol for testing any combination between Cas9 protein and sgRNA to cut the target DNA and may become a routine procedure for any genome editing experiment.

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