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Research Article

The transformation of *dbr2* and *p19* Genes into *Artemisia annua* L. by *Agrobacterium tumefaciens* Mediation: Metabolites Analysis

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Abstract

The low levels of artemisinin in *A. annua* L. are caused by obstacles in its biosynthesis. This study aims to increase artemisinin production through transformation of the *dbr2* and *p19* genes using *Agrobacterium tumefaciens* and evaluate the impact on metabolite production. The *dbr2* and *p19* genes were verified by analyzing PCR products. Plasmids pCAMBIA1303-*dbr2-p19* were transformed into *A. tumefaciens* and subsequently introduced into the leaves from tissue culture of *A. annua* L, using vacuum infiltration and assessed by GUS assay. The transformation in *A. tumefaciens* was successful using a freeze-thaw method. The tissue culture of *A. annua* L. has been infected by *A. tumefaciens* using vacuum infiltration. Based on the GUS histochemical assay, the gene has been successfully inserted in the leaves with an efficiency of 48.2%. The result from UPLC-ESI-MS/MS showed that the level of Artemisinin in the transformation sample with and without pCAMBIA-*dbr2-p19* was detected but not quantifiable while in wild-type leaves were quantified at 0.008% in fresh weight. The AA and DHAA were not detectable but only in the wild type. The transformation was successful, but the quantification of DHAA and AA was unsuccessful because of the low quantity in the samples.

Keywords: *Artemisia annua*; Artemisinin; *Agrobacterium tumefaciens*; *dbr2*; Malaria; *p19*; UPLC-ESI-MS/MS

Transformasi Gen *dbr2* dan *p19* ke *Artemisia annua* L. oleh mediasi *Agrobacterium tumefaciens* serta Analisis Metabolitnya

Abstrak

Rendahnya kadar artemisinin pada *A. annua* L. disebabkan oleh kendala dalam biosintesisnya. Penelitian ini bertujuan meningkatkan produksi artemisinin melalui transformasi gen *dbr2* dan *p19* menggunakan *Agrobacterium tumefaciens* dan mengevaluasi dampaknya pada produksi metabolit. Gen *dbr2* dan *p19* diverifikasi dengan menganalisis produk PCR. Plasmid pCAMBIA1303-*dbr2-p19* ditransformasikan ke *A. tumefaciens*, kemudian ditransfomasikan ke daun-daun dari kultur jaringan *A. annua* L. dari perkecambahan biji steril dengan infiltrasi vakum dan dihitung dengan uji GUS. Transformasi di *A. tumefaciens* berhasil menggunakan metode freeze-thaw. Kultur jaringan *A. annua* L. diinfiltrasi oleh *A. tumefaciens* menggunakan metode vakum. Berdasarkan uji histokimia, gen telah berhasil dimasukkan ke daun dengan efisiensi 48,2%. Hasil pengujian UPLC-ESI-MS/MS menunjukkan bahwa kadar artemisinin dalam sampel transformasi dengan dan tanpa pCAMBIA-*dbr2-p19* terdeteksi tetapi tidak terukur, sementara di daun wild-type sebesar 0,008% dalam berat segar. Senyawa AA dan DHAA tidak terdeteksi dan hanya terdeteksi pada daun wild type. Proses transformasinya telah berhasil, tetapi perhitungan kandungan AA dan DHA tidak dapat dilakukan karena rendanya kadar kedua senyawa pada sampel.

Kata Kunci: Artemisia annua; Artemisinin; Agrobacterium tumefaciens; dbr2; Malaria; p19; UPLC-ESI-MS/MS

1. Introduction

Malaria has still become a global threat, which affected an estimated 247 million cases and 619.000 deaths globally in 2021. Most of the cases (82%) and deaths (95%) averted were in the WHO African Region, followed by the WHO South-East Asia Region (cases by 10% and deaths by 3%).[1] Malaria is caused by a small protozoon from the group of Plasmodium species, and it is divided into several subspecies that cause disease in humans. The genus Plasmodium is an amoeboid intracellular parasite that accumulates malaria pigment, an insoluble metabolite of hemoglobin. Parasites on different vertebrates; some in red blood cells, and some in tissue. It was estimated that around 172 Plasmodium species can infect humans, five species of which are *P. malariae, P. falciparum, P. vivax, P. ovale, and P. knowlesi*. In South-East Asia, the zoonotic malaria, *P. Knowlesi*, is recorded. In humans, these five Plasmodium species cause the disease commonly known as malaria, which is, in Latin, called Malus air or bad air.[2] *P. falciparum* was the cause of the most lethal human malaria.[3] Around the 1970s, Tu you's group discovered the antimalarial drug artemisinin, inspired by the records in traditional Chinese medicine (TCM). Artemisinin is a metabolite derived from the sweet wormwood plant, *A. Annua*.[4] Since its discovery, artemisinin and its derivatives have saved two hundred million malaria patients and become the most effective antimalarial drug.[5]

The obstacle in the development of artemisinin is that *Artemisia annua* is the only source of artemisinin, which resulted in a low yield of only 0.1-1,5% DW (dry weight) of the plant. It was dependent on bacteria, insects, fungi, and climates.[6] The process of extracting artemisinin from *A. annua* is complicated and costly, and it cannot meet the global demand for artemisinin.[7] The chemical synthesis process to produce artemisinin is also challenging due to its unique and complex structure, belonging to the sesquiterpene lactone class and containing an endoperoxide bridge.[8] It also caused the discovery to become less prospective. The new approaches to increase artemisinin production are engineered genetically and through cell culture. There were several primary enzymes in artemisinin biosynthesis, such as FPPS, ADS, CYP71AV1, DBR2, AND ALDH1. Using microorganisms (*Escherichia coli* and *Saccharomyces cerevisiae*) as chassis cells, the heterologous biosynthesis of artemisinin or its precursor through metabolic engineering may provide an alternative source of artemisinin supply.[9] Due to the limited quantities, the enzyme can produce and increase the artemisinin and its precursor in plant cells and yeast.[10] The limitation of secondary metabolites in wild plants encourages the establishment of in vitro cultures, and strategies have been adopted to enhance artemisinin content in these cultures.[11] The gene known as Amorpha-4,11-diene synthase (*ads*) and an anti-silencing gene called *p19* protein (*p19*) were introduced into the leaves of *A. annua* L. plants through a process mediated by *A. tumefaciens.* This genetic transformation of the plant tissue cultures resulted in an increased production of artemisinin, a compound with medicinal properties, in the transformed leaves. Compared to leaves that were not converted, the artemisinin content from the transformed leaves added by combined genes ads (1.4-morpha diena synthase) and *p19* (anti-silencing gene) was boosted 2.57 times.[12] In addition, plant tissue cultures that contain *dbr2* genetically engineered indicated that overexpression of *dbr2* led to the upscaling of artemisinin and direct precursor dihydroartemisinic acid accumulation.[13]

In this study, the *dbr2* gene of *A. annua* L. and the *p19* anti-silencing gene from tomato bushy virus was expressed in transgenic *A. annua* L. plants via *Agrobacterium tumafaciens*, and the investigation of its biological effects on artemisinin and its relative metabolite biosynthesis was evaluated using UPLC-ESI-MS/MS.

2. Method

2.1. Tools

Spectrophotometer UV-VIS (Shimidzu), nanodrop (Thermo Scientific), instrument UPLC (Waters), freezing mikro centrifugation (Eppendorf), polymerase chain reaction (SelectCyclerII), gel doc (Labnet International), analytical balance, pH meter, autoclave, laminar air flow, hot plate, electrophoresis, shaker incubator, shaker at room temperature, UV lamps, desiccator, vacuum pump, micro centrifugation, incubator, water heater, syringe injection, micropipette, refrigerator, freezer -80°C, and all glass equipment in laboratory.

2.2. Materials

2.2.1. Plant Tissue Culture

A. annua L. seeds from Botanical Garden Manako (Lembang, West Java), Murashige and Skoog medium (phytolabs), CleriTM Ultra gelling agent (Himedia), pyridoxine, thiamin, ascorbate acid, glycine, NaOH, HCl, and aquadest.

2.2.2. Agrobacterium Transformation

The study utilized a liquid Yeast Extract Peptone medium, which consisted of 1% NaCl, 1% Bacto-peptone, 0.5% yeast extract, and 1.5% Agar-B as the growth medium. Additionally, 1× TAE buffer was prepared by diluting 50X TAE stock solution, which contained 24.2% Tris base (Promega), 5.71% glacial acetic acid, and 10% EDTA (0.5 M, pH 8.0). Other reagents included a 1 kb DNA ladder (Thermo-Fisher) and agarose (Top Vision, MDBio). Staining for DNA are ethidium bromide and SerRed (ServiceBio). MyTaq Mix (Meridian Bioscience) for the PCR reaction, Forward primers of p19 gene (5'-AAA CTC GAG ATG GAA CGA GCT ATA CAA G-3') and reverse primer (5'-AAA CTC GAG TTA CTC GCC TTCTTT TTC G-3'). Forward primer for dbr2 gene (5'-GCC TAC AAG ATG GGC AG-3') and reverse primer (5'- CTA GAG GAG TGA CCC TTT G-3'). PrestoTM Mini Plasmid Kit (Geneaid) for plasmid isolation. Some antibiotics such as ampicillin and kanamycin also used for this assay.

2.2.3. Plant Transformation

Murashige and Skoog medium (Phytolabs), acetosyringone (Phytolabs), tween-80, cefotaxime, deionized water, Na_2HPO_4, NaH_2PO_4, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$, X-Gluc (Thermo-fisher), triton-X 100, alcohol 96%, alcohol 70%, aquadest.

2.2.4. GUS Histochemical Assay

An 80 mL solution containing phosphate buffer 1M (pH 7.2) Na_2HPO_4, NaH_2PO_4, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$, Triton-X 0.1 mL, and add to 80 mL with sterile deionized water, X-Gluc 41.6 mg dissolved in 8 mL DMSO. For incubation, phosphate buffer and X-Gluc were used with a ratio of 9:1.

2.2.5. UPLC-ESI-MS-MS

Methanol pro analysis (Merck), acetonitrile pro analysis (Merck), aquadest, formic acid, artemisinin standard, artemisinic acid standard, and dihydroartemicinic acid standard (Markherb, Bandung-Indonesia).

2.3. Methods

2.3.1. The preparation *A. annua* L.

A seed of A. annua L. was received from the. It was sterilized using ethanol 70% for 2 minutes and sodium hypochlorite NaOCl 5% for 20 minutes, then rinsed with sterile water three times. The sterilized seeds were cultivated on MS medium pH 6.1 containing vitamins, sucrose 3%, and a gelling agent, which was sterilized by autoclave 121ºC for 15 minutes. The plants received light from lamps 24 hours a day. Then, the plant tissue culture of A. annua L. was sub-culture every 30-35 days.

2.3.2. The preparation of Plasmid Recombinant pCAMBIA-*dbr2-p19* and its confirmation

The glycerol stock containing DH5α with pCAMBIA-*dbr2-p19* was received from the Laboratorium Genetics, Biotechnology and Molecular, School of Life Science, Institute Technology Bandung. The stock was streaked on solid Luria Bertani (LB) medium without antibiotics and then incubated at 37°C overnight. A single colony was taken,

suspended in a liquid LB medium, and shaken overnight at 37°C, 150 rpm. The plasmid from liquid cultures was isolated using a Presto™ Mini Plasmid Kit (PDH100) by Geneaid. The results were measured by nano drops to calculate the plasmid concentration and purity. After that, the plasmid was confirmed by PCR and used specific primers for the *dbr2* and *p19*. The PCR condition [95 °C (5 min) → [95 °C (30 s) → 60 °C (p19) or 46 °C (dbr2) (30 s) → 72 °C (1 min 30 s)] × 25 cycles → 72 °C (7 min)].

2.3.3. The preparation of *A. tumefaciens* AGL1 competence cells

The glycerol stock containing AGL1 was received from the Laboratorium Microbiology, School of Life Science, Institute Technology Bandung. The stock was streaked on a solid YEP medium with appropriate antibiotics and incubated at 28°C for 2 days in dark conditions. A single colony was taken, suspended in a liquid YEP medium, and shaken for 2 days at 28°C, 150 rpm. The liquid culture was transferred in 50 mL YEP medium until OD_{600}=0.4. The culture was placed on ice for 20 minutes. Following by centrifuging at 4°C and 4000 rpm for 10 minutes. After that, the supernatant was discarded, added to 20 ml of cold $CaCl_2$ 0.1 M, and incubated for 30 minutes. Then, centrifuged again at 4°C, 4000 rpm for 10 minutes, the supernatant was discarded and resuspended gently using a micropipette with 2.5 ml of cold $CaCl_2$ 0.1 M with glycerol 15%, aliquoted on a cold microtube for every 100 ul. Lastly, the cell competence was stored at -80°C freezer for transformation.

2.3.4. The transformation of plasmid pCAMBIA1303-*dbr2-p19* into *A. tumefaciens* and its confirmation

Recombinant plasmids were transformed into competence cells of *A. tumefaciens* AGL1. A hundred microliters of *A. tumefaciens* AGL1 were thawed on ice and added 1 µg of plasmid. After that, the plasmid was incubated on ice for 5 minutes and on liquid nitrogen for 5 minutes. Cells were heated at 37°C for 5 minutes and added 1 mL liquid YEP medium and then shaken at 28°C for 4 hours with 150 rpm. Cells were centrifuged at 14.000 rpm for 1 minute and the supernatant was discarded. Pellets were resuspended in 100 µL medium and inoculated in solid YEP containing ampicillin 100 ppm and kanamycin 50 ppm. The plates were incubated for 2 days at 28°C in dark conditions. A single colony was taken and suspended in 10 mL YEP liquid, incubated for 2 days in an incubator shaker at 28ºC and 150 rpm. The liquid culture was isolated using the Plasmid Isolation Kit (ServiceBio). The result was measured by NanoDrop and used for PCR confirmation. After transformation, the PCR product from plasmid *A. tumefaciens* AGL1 was characterized using electrophoresis 1% agarose and a specific primer of the *p19* gene and *dbr2*. The PCR condition was applied as follows: [95 °C (5 min) → [95 °C (30 s) → 60 °C (*p19*) or 46 °C (*dbr2*) (30 s) → 72 °C (1 min 30 s)] × 25 cycles → 72 °C (7 min)].

2.3.5. The transformation of plasmid pCAMBIA1303-*dbr2-p19* into *A. tumefaciens* and its confirmation

Overnight culture of *A. tumefaciens* AGL1 with and without pCAMBIA1303-*dbr2-p19* was prepared in 50 mL YEP medium until OD_{600}=1 and pelleted, then mixed in MS 0.5 medium and shaken at 150 rpm and at room temperature for 4 hours. The acetosyringone 200 mM and Tween-80 0.02% were added to the mixture. The plant *A. annua* L. leaves have added around 20-30 leaves. The mixture was vacuumed for 45 min at room temperature. After that, all the leaves were placed in MS co-cultivation for 3 days in dark conditions. Then, it was washed in cefotaxime 300 ppm, rinsed 3 times, and dried. Apart from the vacuum infiltration, leaves were used for analysis using GUS staining. Around 3 leaves were incubated in dark conditions for several days, and then the chlorophyll was washed with alcohol 96% for 24 hours. The successful transient transformation of the leaves gave them a blue color, and it was analyzed using the software Image.

Efficiency of transformation = a / b x 100%

a = width of the blue area
b = total leaf area

2.3.6. The analysis of Artemisinin, Dihidroartemisinic Acid (DHAA), and Artemisinic Acid (AA) Content

Hundreds of wild-type and transformed fresh leaves from *A. annua* L. were extracted by maceration in 1 mL methanol. The supernatants were collected and filtered using a sterile filter of 0.22 µm. Then, liquid extract content like artemisinin, artemisinic acid (AA), and dihydroartemisinic acid (DHAA) were analyzed by UPLC-ESI- MS/MS. For the mobile phase, a mixture of solution 1 (aquadest with 0.1% formic acid) and solution 2 (acetonitrile with 0.1% formic Acid) is used, while the stationary phase used is BEHC18 (Waters), and the analysis time for each sample is 20 minutes.

3. Result and Discussion

The results of this process show that germination starts on the 7th day and develops into complete plants by the 32nd day (Figure 1A). After the 35th day, all the grown plants are transferred to fresh MS medium until they reach a larger size. They are ready to be used as materials for the transformation process with A. tumefaciens containing the targeted gene (Figure 1B).

A key enzyme in the artemisinin biosynthetic pathway, *dbr2* (artemisinic aldehyde Δ11(13) double bond reductase) was combined with the synthetic *p19* gene from tomato bushy stunt virus. The *p19* gene functions in disturbing plant protection as post-transcriptional gene silencing.[14] These two genes have been amplified with PCR using Mytaq Master Mix DNA Polymerase. The glycerol stock of Escherichia coli DH5α which contains pCAMBIA1303-*dbr2-p19* was streaked on Luria Bertani (LB) medium and incubated at 37ºC overnight. The single colony was selected and grown overnight on LB liquid medium, incubated again at 37ºC, and shaken at 150 rpm. The plasmid from the culture was isolated. After that, the result of the plasmids was measured using nano drops to calculate their concentration and purity. The results were positive where the isolate contained 22.9 ng/µg and 44.5 ng/µg of plasmids and pure. The gen of *dbr2* and *p19* were confirmed using PCR and specific primer. The annealing time for the *dbr2* gene was 46ºC and 60ºC for the *p19* gene. The PCR products were analyzed using electrophoresis while ethidium bromide visualized the DNA. The results showed that the gene *dbr2* was confirmed by electrophoresis, which showed a band around 1000 bp (Figure 1C) while the actual band was 1164 bp based on previous research but still unpublished. From another researcher, we found that the length of *dbr2* gene was 919 bp.[15] Moreover, the *p19* gene was also contained in the plasmid, which confirmed bands around 500 bp (519 bp) (Figure 1D). Due to the positive result in this plasmid, it can be transferred into *A. tumefaciens* strain AGL1.

Plant genetic modification extensively depends on *A. tumefaciens,* a bacterial pathogen, as an effective means to introduce desired genes into a host plant. This process involves the transfer of T-DNA, which can integrate into the plant genome within the nucleus, allowing for inheritance in subsequent generations (known as stable transformation). Alternatively, the exogenous genetic material may temporarily reside within the cell nucleus, devoid of genomic integration, while being subject to transcription to produce desired gene products. This process is commonly denoted as transient transformation.[16] *A. tumefaciens* AGL1 strain was the most effective to be transformed into *A. annua* than GV3101 and LBA4404 strain.[17] The competence cells of A. tumefaciens AGL1 were created using the $CaCl_2$ method, where the concentration was 100 mM. This method is a cost-effective and uncomplicated procedure that does not require any specialized equipment.[18] The *A. tumefaciens* AGL1 in the YEP liquid medium reached OD_{600}=0.4. This value was critical because the transformation's effectiveness depends on the bacterial growth phase. The log phase was the best choice.[19] In an LB

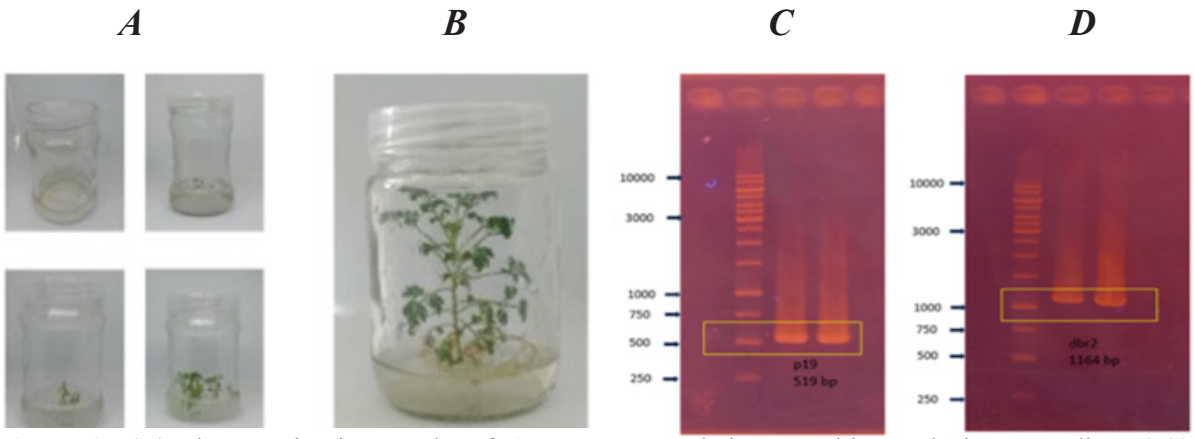

Figure 1. (A) The germination results of *A. annua* L. seeds in Murashige and Skoog Medium 4.43 g/L and enriched with Sukrosa 3%, Myo-inositol 100 mg/L, Pyridoxin 0.5 mg/L, Thiamin 0.1 mg/L, Nicotinic Acid 0.5 mg/L, and Pyridoxin 2.0 mg/L. It showed that the seeds' sterilization process was successful, and their growing condition was excellent. (B) *A. annua* L. plant obtained from the subculture. PCAMBIA1303-*dbr2-p19* could transform this plant via *A. tumefaciens* which contained the plasmid. Electropherogram of the confirmation dbr2 (C) and p19 (D) genes in the plasmid (L = DNA ladder 1 kb. 1 and 2= plasmid isolation result). It showed that the *dbr2* gene was confirmed in the band around 1000 bp and the *p19* gene at 500 bp. This result confirmed that the isolated plasmid could be transformed into *A. tumefaciens* AGL1.

liquid medium containing ampicillin 100 ppm, the *A. tumefaciens* AGL1 strain needs around 12 hours to reach OD_{600}=0.4 (Table 1). It was recommended that YEP medium be used instead of LB medium because YEP is more suitable and nutritious for A. tumefaciens. Based on previous research, YEP medium was more effective due to peptone, yeast extract, and sodium chloride composition. These provide organic sources like nitrogen, amino acids, and minerals for bacteria growth. These compounds possibly gave more valuable nutrition for the bacteria compared to LB with tryptone and YEM with mannitol composition.[20]

A hundred microliter of *A. tumefaciens* AGL1 competence cells were transformed with isolate plasmid, which contained pCAMBIA1303-*dbr2-p19*. The transformation was done by a freeze-thaw method. After transformation based on kanamycin selection, the kanamycin resistance gene harboring in pCAMBIA1303 showed a positive result. A single transformed colony of *A. tumefaciens* AGL1 survived in YEP medium with ampicillin 100 ppm and kanamycin 50 ppm (Figure 2A).

This colony was confirmed by PCR analysis using a specific primer of the *dbr2* gene, and the electropherogram showed a band around 1000 bp compared to DNA ladder 1 kb and positive control (isolate plasmid before transformation to AGL1.The single colony from the transformation result was subcultured in a YEP liquid medium with ampicillin 100 ppm and kanamycin 50 ppm. After incubation for 2 days at 28ºC in dark conditions, the culture was used for plasmid isolation and confirmed by PCR using a specific primer of the *p19* gene. The electropherogram showed colonies harboring the gene *p19* (Figure 2B). From its confirmation, the AGL1 containing pCAMBIA1303-*dbr2-p19* was able to transform in the plant tissue culture of *A. annua* L.

The transformation of *A. tumefaciens* AGL1 recombinants with pCAMBIA1303-*dbr2-p19* and AGL1 non-recombinants into *A. annua* leaves were collected using 45 minutes of vacuum infiltration method.[21]

Table 1. The calculation of OD_{600} value by time.

Times (hours)	4	5	7	8	9	10	11	12	12.5
OD_{600}	0.043	0.075	0.083	0.130	0.151	0.219	0.284	0.349	0.403

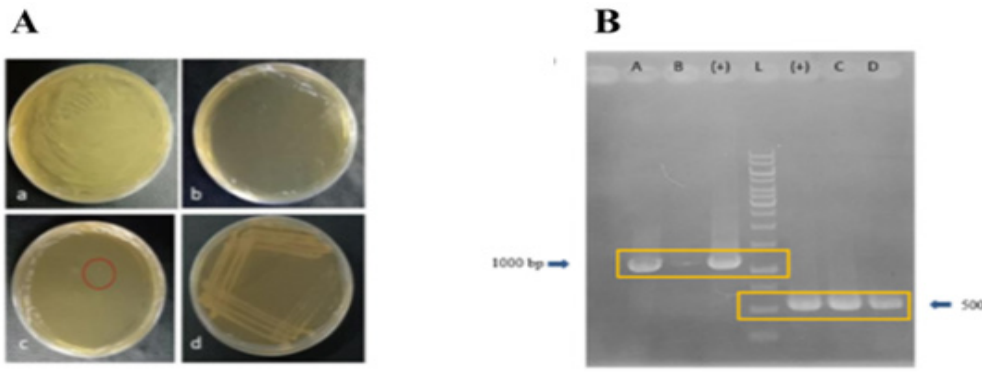

Figure 2. (A) Medium selection for transformation in AGL1. a = AGL1 without plasmid in YEP with Ampicillin 100 ppm, b= AGL1 without plasmid in YEP with Ampicillin 100 ppm and Kanamycin 50 ppm, c and d = AGL1 with pCAMBIA1303-dbr2-p19 in YEP with Ampicillin 100 ppm and Kanamycin 50 ppm. (B) Electropherogram of the confirmation *dbr2* and *p19* gene in plasmid isolation product. (L = DNA ladder 1 kb. (+) plasmid isolation before transformation to A. tumefaciens AGL1. A and B PCR product of plasmid isolation result after transformation. C and D = PCR product of plasmid isolation result after transformation). It showed that dbr2 gene was confirmed in a band around 1000 bp, while *p19* gene was detected at 500 bp.

Several research projects have demonstrated the efficacy of this approach in enhancing the frequency of *A. tumefaciens* expression in plants. Vacuum infiltration removes air from the intracellular space, allowing the medium to penetrate. This method is mainly chosen when transferring genetic material to small A. annua leaves related to this research's leaf sample, produced by seed growth in sterile media. The infected plants were then incubated for three days to optimize transient transformation in plants using MS medium and kept in dark condition, at room temperature. After 3 days, 3-4 leaves were taken, incubated in GUS buffer staining solution, and incubated for several days. After the incubation, the leaves were rinsed using alcohol 96% to leach the chlorophyll. After 24 hours, the chlorophyll disappeared from the leaves. The successful transient transformation left a blue color on its leaf surface.

The leaves infected with AGL1 harboring pCAMBIA1303-*dbr2-p19* showed a blue color (A), which means that the transient transformation was successful when compared to the leaves infected only with AGL1 (B) and the wild-type (C) in Figure 3. This is because the pCAMBIA plasmid contains the GUS reporter gene, which reacts with the X-Gluc buffer, resulting in a blue color.[22] On the other hand, AGL1 without the plasmid and the wild type do not contain the GUS reporter gene hence they do not visualize the blue color. Due to the blue color not being too strong, this transient transformation's efficiency was 48.2% (Table 2). It means that the additional surfactants that decrease the surface tension from the leaves' surface needs to be re-evaluated. In this research, we used tween-80, but from the previous research, the efficiency of transformation using surfactant silweet-77 and silweet-408 gave higher efficiency and better transformation.[17]

Sample preparation for the quantification of Artemisinin, Artemisinic Acid (AA), and Dihydroartemisinic Acid (DHAA), involved the process of maceration using 1 mL of analytical-grade methanol as the solvent. The leaf samples used in the study were derived from three distinct treatments, consisting of leaves infiltrated with A. tumefaciens AGL1 carrying pCAMBIA1303-*dbr2-p19*, leaves infiltrated with *A. tumefaciens* AGL1, and the corresponding wild-type control. Notably, the leaf samples were carefully collected in their fresh state to preserve their natural composition without undergoing any prior drying process. To ensure accuracy and reproducibility, the sample preparation procedure was carried out with meticulous attention, and the process was replicated three times.

Subsequently, the methanolic extracts

Figure 3. Transient transformed leaves of A. annua L. with A. tumefaciens using vacuum, AGL1 with pCAMBIA1303-dbr2-p19 (A), which report GUS protein (blue color), AGL1 without plasmid (B) and wild-type (C) (without blue color).

obtained from maceration were subjected to filtration to remove any particulate matter and impurities. The filtrates were then introduced into the UPLC-ESI-MS/MS instrument for analysis, which enabled sensitive and precise measurement of the target compounds. This instrument was suitable because the sample collected from this research was limited and the UPLC LOD and LOQ values are lower than HPLC.[23] For the quantification of Artemisinin, DHAA, and AA, a well-established UPLC-ESI-MS/MS method, previously designed and rigorously validated by researchers, was employed. This analytical method included the use of appropriate standard compounds, enabling accurate determination of the concentrations of the target analytes in the leaf samples.

Based on the measurements conducted using UPLC-ESI-MS/MS, it was observed that the levels of artemisinin (282 Da) were detectable in all three types of samples (Figure 5A-C). However, quantifiable levels of artemisinin were only found in the wild-type sample, amounting to 0.008%. As a result, the comparison between the levels of artemisinin in the transformed and non-transformed samples cannot be accurately determined. Furthermore, for the measurement of AA (Artemisinic Acid, 234 Da) and DHAA (Dihydroartemisinic Acid, 236 Da), they were not detectable in the samples transformed with A. tumefaciens AGL1, both with and without the plasmid.

However, these compounds were detectable but not quantifiable in the wild-type leaves. The low levels of artemisinin and the absence of DHAA and AA detection in the methanolic extracts of A. annua L. leaves after transformation might be attributed to events occurring during the co-cultivation process in 3 days, which takes place after the vacuum infiltration step. The injuries inflicted before the transformation process to facilitate A. tumefaciens attachment might also have an impact. Notably, when the transformed leaves were extracted with methanol, they exhibited a brownish-yellow color, whereas the wild-type leaves displayed a dark green color. This showed signs of senescence.[24] Based on previous research, during the early stages of the A. annua crop growth, the combined levels of artemisinin and its precursors were more elevated in green leaves compared to dead leaves.[25] So, the co-cultivation process after vacuum infitration should be shorter.

The results do not align with the theoretical expectations based on the analysis of Artemisinin, DHAA, and AA levels using UPLC-ESI-MS/MS. Therefore, further evaluation is needed, such as carrying out the insertion of the pCAMBIA1303-*dbr2-p19* plasmid until the stage of stable transformation. Using the agrobacterium suspension soaked in a pollen tube, the new gene could be inherited to the new plant.[26]

Table 2. The calculation of transformation effectiveness

Type of the leave sample	1	2	3	Average
Transformed by AGL1 pCAMBIA1303-dbr2-p19	52.91%	46.27%	45.41%	48.20%
Transformed by AGL1 without plasmid	0	0	0	0
Wild type	0	0	0	0

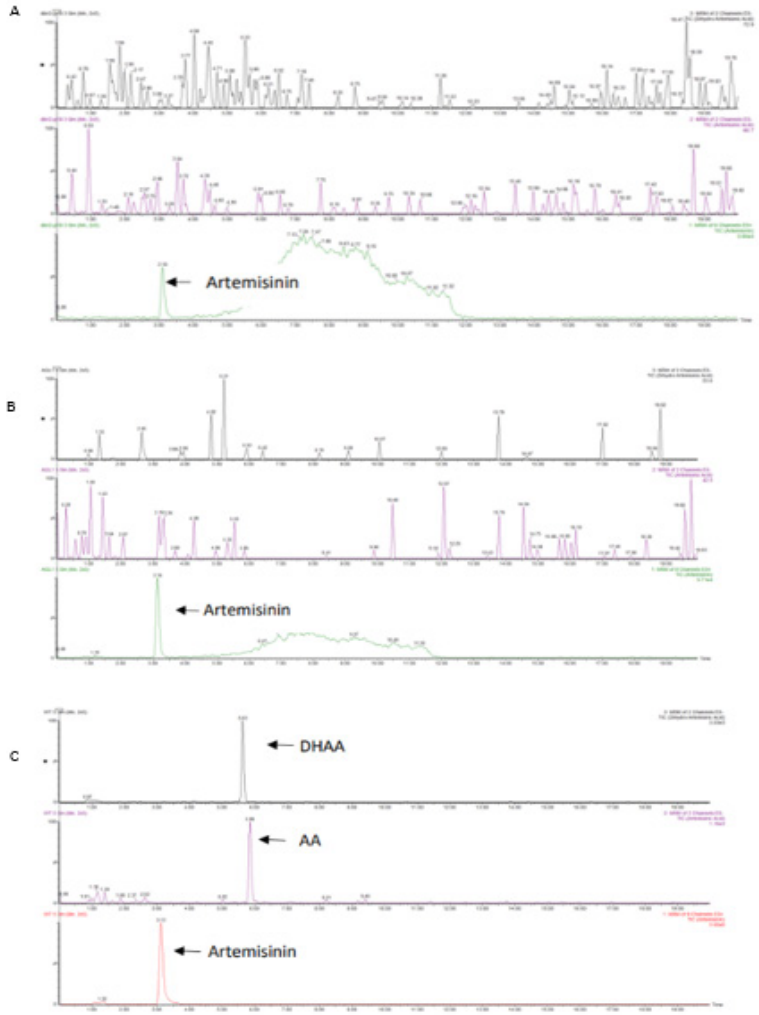

Figure 4. The chromatogram results for Multiple Reaction Monitoring (MRM) of the compounds DHAA, AA, and Artemisinin in the methanolic extract of A. annua L. (A) harboring pCAMBIA1303-dbr2-p19 leaves. UPLC-ESI-MS/MS did not detect the DHAA and AA contents. Meanwhile, the Artemisinin content was detected (tR 3.00). (B) infected by AGL1 without plasmid. UPLC-ESI-MS/MS did not detect the DHAA and AA contents. The Artemisinin content was detected (tR 3.00). (C) A. annua L. leaves wild type. The DHAA (tR 5.50) and AA (tR 5.70) contents were detected by UPLC-ESI-MS/MS but not quantified. The Artemisinin content was quantified 0.008% (tR 3.00).

This additional step may help address the discrepancies and obtain more accurate and consistent results in analyzing the target compounds.

4. Conclusion

In conclusion, our research successfully transformed the pCAMBIA1303-*dbr2-p19* plasmid into *A. tumefaciens* AGL1 and subsequently introduced it into the plant tissue culture of *A. annua* L. This transformation led to the detection of artemisinin in the transformed leaf samples, indicating the successful incorporation of the desired plasmid and its impact on the artemisinin content within the plant tissues. However, the absence of detectable levels of DHAA and AA in the transformed samples warrants further investigation into the factors influencing their biosynthesis. These findings lay a solid foundation for future studies, emphasizing the need to unravel the complexities of artemisinin biosynthesis in *A. annua* L. plants, with potential implications for advancing medicinal research and biotechnological applications.

Reference

1. WHO. World malaria report. Geneva: World Health Organization; 2022.

2. Talapko J, Škrlec I, Alebić T, Jukić M, Včev A. Malaria: The past and the present. Microorganisms. 2019;7(6).
3. ellems TE and Plowe C V. Chloroquine-Resistant Malaria. Journal of Infectious Diseases. 2001;184(6): 770-6.
4. Tu Y. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. Nature Medicine. 2011;17(10): 1217–20.
5. Rathmes G, Rumisha SF, Lucas TCD, Twohig KA, Python A, Nguyen M, et al. Global estimation of anti-malarial drug effectiveness for the treatment of uncomplicated Plasmodium falciparum malaria 1991–2019. Malaria Journal. 2020;19(1): 1–15.
6. Barbacka K, Baer-Dubowska W. Searching for Artemisinin Production Improvement in Plants and Microorganisms. Current Pharmaceutical Biotechnology. 2011;12(11): 1743–51.
7. Bouwmeester HJ, Wallaart TE, Janssen MHA, Van Loo B, Jansen BJM, Posthumus MA, et al. Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry. 1999;52(5): 843–54.
8. Knudsmark Jessing K and Duke SO, Cedergreeen N. Potential Ecological Roles of Artemisinin Produced by Artemisia annua L. Journal of Chemical Ecology. 2014;40(2): 100–17.
9. Zhao L, Zhu Y, Jia H, Han Y, Zheng X, Wang M, et al. From Plant to Yeast—Advances in Biosynthesis of Artemisinin. Molecules. 2022;27(20).
10. Muangphrom P, Seki H, Fukushima EO, Muranaka T. Artemisinin-based antimalarial research: application of biotechnology to the production of artemisinin, its mode of action, and the mechanism of resistance of Plasmodium parasites. Journal of Natural Medicines. 2016;70(3):318–34.
11. Ali M, Abbasi BH, Ahmad N, Khan H, Ali GS. Strategies to enhance biologically active-secondary metabolites in cell cultures of Artemisia–current trends. Critical Reviews in Biotechnology. 2017;37(7): 833–51.
12. Elfahmi E, Cahyani FM, Kristanti T, Suhandono S. Transformation of amorphadiene synthase and antisilencing P19 genes into artemisia annua L. And its effect on antimalarial artemisinin production. Advanced Pharmaceutical Bulletin. 2020;10(3): 464-71.
13. Zhang Y, Zhou WE, Yan JQ, Liu M, Zhou Y, Shen X, et al. A review of the extraction and determination methods of thirteen essential vitamins to the human body: An update from 2010. Molecules. 2018;23(6):1–25.
14. Canto T, Uhrig JF, Swanson M, Wright KM, MacFarlane SA. Translocation of Tomato Bushy Stunt Virus P19 Protein into the Nucleus by ALY Proteins Compromises Its Silencing Suppressor Activity. Journal of Virology. 2006;80(18): 9064–72.
15. Yuan Y, Liu W, Zhang Q, Xiang L, Liu X, Chen M, et al. Overexpression of artemisinic aldehyde Δ11 (13) reductase gene-enhanced artemisinin and its relative metabolite biosynthesis in transgenic Artemisia annua L. Biotechnology and Applied Biochemistry. 2015;62(1):17–23.
16. Hwang HH, Yu M, Lai EM. Agrobacterium -Mediated Plant Transformation: Biology and Applications . The Arabidopsis Book. 2017;15:e0186.
17. Elfahmi, Suhandono S, Chahyadi A. Optimization of genetic transformation of Artemisia annua L. Using Agrobacterium for Artemisinin production. Pharmacognosy Magazine. 2014;10(37):S176–80.
18. Chang AY, Chau VWY, Landas JA, Pang Y. Preparation of calcium competent Escherichia coli and heat-shock transformation. Journal of Experimental Microbiology and Immunology. 2017;1(June):22–5.
19. Ma R, Yu Z, Cai Q, Li H, Dong Y, Oksman-Caldentey KM, et al. Agrobacterium-mediated genetic transformation of the medicinal plant veratrum dahuricum. Plants. 2020;9(2):1–12.
20. Iqbal A, Dave N, Kant T. Comparative Analysis of Different Nutrient Media for Growth of Agrobacterium tumefaciens under Small Volume Cultures. 2021;24(2):27–33.
21. Elfahmi E, Cahyani FM, Lubis ADM,

Kristanti T, Suhandono S. Transient Transformation of Artemisinic Aldehyde Δ 11 (13) Double Bond Reductase (dbr2) Gene into Artemisia annua L. Indonesian Journal of Biotechnology. 2016;21(2):76-85.
22. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO journal. 1987;6(13): 3901–7.
23. Klimczak I, Gliszczynska-Świglo A. Comparison of UPLC and HPLC methods for determination of vitamin C. Food Chemistry. 2015;175: 100–5.
24. Burman N, Chandran D, Khurana JP. A Rapid and Highly Efficient Method for Transient Gene Expression in Rice Plants. Frontiers in Plant Science. 2020;11: 584011.
25. Lommen WJM, Schenk E, Bouwmeester HJ, Verstappen FWA. Trichome dynamics and artemisinin accumulation during development and senescence of Artemisia annua leaves. Planta Medica. 2006;72(4): 336–45.
26. Bélanger JG, Copley TR, Hoyos-Villegas V, Charron JB, O'Donoughue L. A comprehensive review of in planta stable transformation strategies. Plant Methods. 2024;20(1): 1–24.