

Transient Transformation of Artemisinic Aldehyde Δ 11 (13) Double Bond Reductase (*dbr2*) Gene into *Artemisia annua* L.

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Abstract

Global demand of antimalarial drug artemisinin has a gap with production capacity from existing sources since the low content of this compound from *Artemia annua* L. Genetic engineering-based strategy for *A. annua* plant on key enzymes in artemisinin biosynthetic pathway is needed. Artemisinic aldehyde Δ 11 (13) double bond reductase (*dbr2*) is one of the key enzyme on artemisinin biosynthesis which was studied in this research. *Agrobacterium tumefaciens*-mediated transformation of *A. annua* using *dbr2* was carried out. Synthetic *dbr2* was ligated into pCAMBIA1303 and transformed into *Escherichia coli* DH5 α . pCAMBIA1303-*dbr2* plasmid was transformed to *A. tumefaciens* AGL1. Leaves of *A. annua* were infected by positive transformant of recombinant *A. tumefaciens* (OD₆₀₀ \approx 1) supplemented with acetosyringone 50 ppm, and Silwet S-408 0.02%. Samples were incubated in desiccators connected with vacuum pump, this method is called infiltration vacuum. Leaves were covered in dark for 45 min, and co-cultivated on MS co-cultivation media for 3 days. All leaves were washed in 300 ppm cefotaxime and divided into 2 parts; 3 leaves for GUS histochemical assay and 300 mg of leaves for HPLC analysis. Transient transformation was done in triplicate. In GUS histochemical assay, pCAMBIA1303 and pCAMBIA-*dbr2* showed positive blue spot where coefficient of variance was less than 5%. PCR analysis for genomic DNA of transformed *A. annua* showed a positive result of inserted *dbr2* recombinant indicated by migration profile and direct sequencing analysis. It could be concluded that pCAMBIA-*dbr2* construct and transformation into *A. annua* have been successfully performed.

Keywords: *Artemisia annua* L., artemisinin, construction, malaria, pCAMBIA-*dbr2*, transient

Introduction

Malaria has already existed since long time ago and become endemic in the tropical and subtropical countries, particularly in South East Asia region including Indonesia. Chloroquine resistant followed by sulfadoxine-pyrimethamine resistant had came up at the end of 1900s causing malaria prevalence increases every year. In early 2000s, Indonesia's Ministry of Health recorded

15 million cases of malaria and 38,000 died every year (Depkes RI, 2008), also that 262 million cases and 839,000 people were death globally (WHO, 2015) due to medical failure. In 2001, World Health Organization issued ACTs (Artemisinin based-combined therapies) policy as first-line in malaria treatment caused by drug resistance, firstly tried in Africa region and spread after to others endemic area in the world (WHO, 2015). As the result, mortality number could decrease into 0.92%, 0.42%, to 0.2% from 2005 to 2007 (Depkes RI, 2008).

Indonesia used two ACTs drugs, artesunate-amodiaquine and dihydroartemisinin-piperazine. These drugs contain artemisinin derived compound which has

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antimalarial activity against *Plasmodium* sp. parasite at endoeritrocytic stage. Artemisinin was found in *Artemisia annua* L. plant which has 0.05–0.2% dried content (Dewick, 2009) thus inhibit commercial production in common. Many efforts have been made such as semisynthetic to enhance artemisinin production as base compound for ACTs. However peroxide bridge structure was unique and hard to be made with common chemical reaction, therefore artemisinin biosynthetic engineering in *A. annua* plant was focused for five key enzymes (Brown, 2010). Artemisinic aldehyde Δ 11 (13)double bond reductase (*dbr2*) is one of key enzymes which has important role as check point in the path separation to artemisinin. The aim of this study was to transform the *dbr2* gene in *A. annua* leaves explant mediated by *A. tumefaciens*.

Materials and Methods

Materials

Gene cloning

Escherichia coli DH5 α strain; LB med *E. coli* DH5 α strain; LB medium; *A. tumefaciens* AGL1 strain; YEP medium; CCMB80 buffer; TSS buffer; CaCl₂ buffer 20 mM; antibiotic selection (kanamycin, rifampicin, and ampicillin); liquid nitrogen; synthetic *dbr2* gene plasmid BioBasic Inc. (BBI No. ITB001-2).

Gene construction and confirmation

Restriction Enzymes: *Bgl*III Promega, *Spe*I Promega, *Nhe*I Thermo Scientific, *Sac*I Thermo Scientific; *B*Buffer™ Promega; 1x *Tango Buffer*™ Thermo Scientific; T4 DNA

Buffer and Enzyme Ligation Thermo Scientific; Gel/PCR Purification kit Geneaid; Sequencing Primers (Table 1).

PCR analysis

Dream Taq™ Green Master Mix; nuclease-free water; 6x Loading Dye DNA Thermo Scientific; Mass ruler DNA™ Ready-Mix Thermo Scientific; Gene ruler1kb™ Thermo Scientific; Reaction Primers (Table 2). PCR system for *dbr2* specific (after optimizing) using 25 cycle, with 95°C for 3 min pre and 30s, 46°C for 30s, and 72°C for 1.5 min and 7 min extension, then CaMV35S – GUS PCR system using 25 cycle, with 95°C for 3 min pre and 30s, 60°C for 30s, and 72°C for 1.5 min and 7 min extension.

Plant transformation

A. annua from Tawangmangu, Central Java, Indonesia; ethanol 70%; sodium hypochlorite 0.75%; acetosyringone; Silwet S-408; cefotaxime; mediums (Table 3).

GUS histochemical assay

For 100 mL solution contain: X-Gluc 52.5 mg, phosphate buffer 1M (pH 7) 5 mL, Triton-X 0.1 mL, and add to 100 with Aqua Deion Steril.

Gene isolation

Reagent for plasmid isolation containing: Solution 1 [glucose 50 mM, Tris Cl 25 mM, EDTA (pH 8) 10mM], Solution 2 [NaOH 0.2 N, SDS 2% w/v], Solution 3 [NaOAc5 M, Glacial acetic acid], isopropanol, ethanol 70%, and TE-RNase; Plant Genomic Isolation kit Geneshun.

Table 1. Primer sequences for sequencing analysis.

Primer Name	Sequence
Primer_DBR_Forward	5'-GCCTACAAGATGGGCAAG-3'
Primer_3_DBR_Reverse	5'-TTGTGAGCTGCATCAACCAC-3'
Primer_CaMV_35S_Forward	5'-ATAGAGGACCTAACAGAACTCG-3'
Primer_3_GUS_Reverse	5'-TTCCACAGTTTTTCGCGATCC-3'

Table 2. Primer specific sequences.

Primer Name	Sequence
dbr2F	5'-GCCTACAAGATGGGCAAG-3'
dbr2R	5'-CTAGAGGAGTGACCCTTTG-3'
FCaMV_35S	5'-ATAGAGGACCTAACAGAACTCG-3'
RGUS	5'-TTCCACAGTTTTTCGCGATCC-3'

Table 3. Plant medium composition.

Medium	Composition
Co-cultivation	MS Salt full, Sucrose 3%, Agar 0,8% (pH = 5.8)
Infection	½ MS Salt, Sucrose 3% (pH = 5.8)

Methods

Plasmid construction

A good sample of pCAMBIA133 and pUC57-*dbi2* vector was chosen by migration profile from the result of polymerase chain reaction confirmation. Those vector were set as templates for restriction reaction with *Bgl*I and *Spe*I enzymes. The restriction reactions were separated by 1% agarose gel electrophoresis and cut at the desired band size, then purified it with Gel/PCR Purification kit Geneaid. All elution liquids collected from same sample were pooled in one tube, then defined the final concentration using Multidrop Thermo Scientific Multiskan Go™ followed by checking the existence of desired fragment size with gel electrophoresis. Gen and vector were ligated with T4 DNA ligation enzyme, incubated at 22°C for an hour then all of reaction were introduced into *E. coli* DH5α competent cell and transferred to LB medium with antibiotic selection, incubate at 37°C overnight (Table 4).

Confirmation of plasmid

All positive transformants were grown overnight in LB medium with antibiotic selection. Recombinant plasmids were isolated using solution 1 (GTE solution), 2 (NaOH – SDS solution), and 3 (KOAc solution), then ends up DNA pellet reconstituted with TE-RNase. Plasmid solution was diluted to 20x factor. Furthermore plasmids were confirmed by migration analysis with following procedures put 5 µL of plasmid into 0.8% agarose gel and run electrophoresis 30 minute, 100V; PCR analysis in following procedures: set PCR reaction with *dbi2* specific primers and run the system as describe above. Screen the result in 1% agarose gel electrophoresis; restriction analysis with following procedures: set restriction reaction with *Nhe*I and *Sac*I enzyme incubate 3 hours at 37°C. Separate by 0.8% agarose gel electrophoresis, and

analyze the fragment size pattern; and DNA Sequence Analysis with following procedures 20 µL plasmid (undiluted) was send to Macrogen Inc., South Korea, to take sequence analysis with existing primer for sequencing.

Cloning the plasmids in *Agrobacterium tumefaciens*

Chemically competent cell of *A. tumefaciens* AGL1 were prepared using liquid nitrogen method (Chen *et al.*, 1994), then pCAMBIA-*dbi2* plasmid were transformed into the competent cell with YEP medium (+ rRifampicin 50 ppm, ampicillinA 100 ppm, and kKanamycin 50 ppm). The recombinant cells were incubated for three days covered in dark at room temperature. Positive colonies were analyzed with crude PCR with CaMV35s – GUS PCR system.

Transient transformation of pCAMBIA1302-*dbi2* into *Artemisia annua* L.

Hundreds mg leaves explant near shoot apical were weighed and sterilized with ethanol 70% for 1 min, sodium hypochlorite 0.75% for 15 min, then rinsed 3 times with sterilized aquadest for 5 min. Overnight culture ($OD_{600} \approx 1$) of positive *A. tumefaciens* transformants was pelleted, then mixed with sterile explant in MS medium, acetosyringone 50 ppm, and Silwet S-408 0.02%. The mixture was vacuum for 45 min in dark room temperature, and it was placed in MS-co-cultivation in 3 days covered in dark. After that it was washed in cefotaxime 200 ppm, rinsed 3 times and dried.

Analysis of gene in transformed *Artemisia annua* L.

A few leaves were vacuumed in GUS staining solution then store in room temperature 3 days and measure blue spot area with ImageJ software. Another leaves were prepared for genomic isolation with Plant Genomic Isolation kit Geneshun. Genomic samples were set in PCR reaction with FCaMV35S and RGUS primer and run system as described above.

Table 4. pCAMBIA-*dbr2* ligation reaction composition.

	Initial Concentration	Final Concentration	Sample	Ligation Control
Nuclease-free water	-	-	8.8 μ L	16.8 μ L
<i>dbr2</i> fragment	23 ng/ μ L	\pm 160 ng	8 μ L	-
pCAMBIA1303 fragment	94 ng/ μ L	\pm 100 ng	1 μ L	1 μ L
T4 DNA Ligase Buffer	10x	1x	2 μ L	2 μ L
T4 DNA Ligase Enzyme	5 Weiss Unit / μ L	1 Weiss Unit	0.2 μ L+ 20 μ L	0.2 μ L+ 20 μ L

Results and Discussion

Plasmid samples in stock were checked by gel electrophoresis to ensure quality and intensity of plasmid band. The presence of DNA ladder was being as a reference of samples migration with each other. A bold and clear band with high intensity like plasmid from C, D, E, and F (Figure 1) indicated a good plasmid. In order to improve efficiency and effectiveness of restriction reaction, a double digest method were done using Buffer B™ Promega with 2-fold *Bgl*III concentration consequently. This could reduce purification step which tend to increase the concentration at last. pC1 and pC2 fragments could be preconcerted with a complete restriction because of two enzymes have managed to cut pUC57-*dbr2* plasmid in 2 fragments as desired, since the distance between two restriction sites just 7 bp (out of gel). To obtain high final concentration after purification, total elution buffer was reduced then two or more same fragment gels were purified and collected altogether into one tube, which pC1

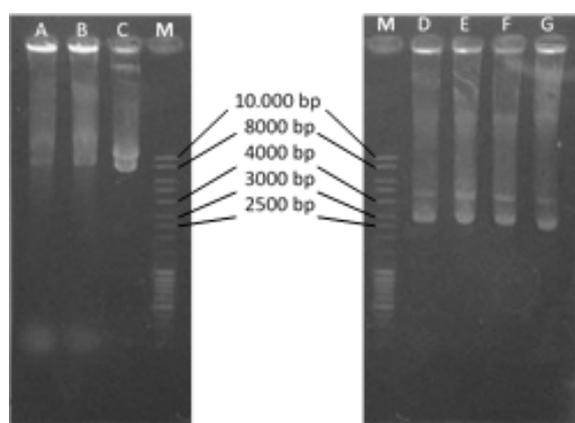


Figure 1. Electrophoregram of A - C = pCAMBIA1303, D - G = pUC57 - *dbr2* migration relative to M = DNA ladder.

with pC2 and D1, D2, D3, with D4 (Figure 2). All purified samples then were checked by its existency (boldness and clearness of fragment band) and quality (concentration and purity) to ensure a fragment condition after purification (Table 5). The purification results were a good with bold and clear band also quite high of concentration and purity (Figure 3), thus the ligation reaction with high success rate would be achieved. Composition of ligation 1:8 (pCAMBIA1303:*dbr2*) was used regard to the amount of insert (*dbr2*) should be more than vector (pCAMBIA1303). Based on the comparison of concentration value, *dbr2* fragment has to be 8 times more than pCAMBIA1303 to make an effective collision in reaction. After one hour incubation at 22°C temperature, all samples were transformed into *E. coli* DH5 α with suitable antibiotic selection.

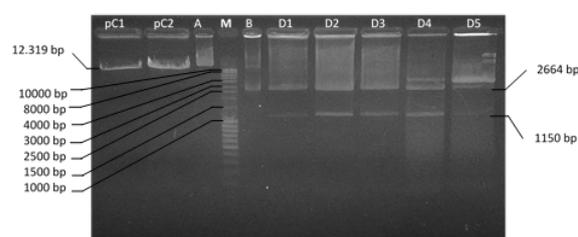


Figure 2. Electrophoregram of restriction fragment size of pC1 and pC2 = pCAMBIA1303, D1-D5 = pUC57 - *dbr2* with *Bgl*III and *Spe*I compared to uncut A = pCAMBIA1303, B = pUC57-*dbr2* plasmid, DNA ladder (M).

Table 5. Absorbance measurement of purified fragment at 260 nm and 280 nm..

Maximum Wavelength	Absorbance		
	Blank	pCAMBIA1303	<i>dbr2</i>
260 nm	0.058	0.156	0.082
280 nm	0.051	0.101	0.063
Concentration (ng/ μ L)		94.230	23.080
Purity (A_{260}/A_{280})		1.960	2.000

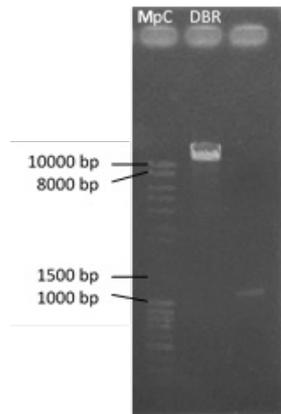


Figure 3. Electrophoregram result to check the presence and size conformity of pC = pCAMBIA1303 and DBR = *dbr2* fragment.

Figure 4 showed that all transformation process were successful due to clean negative transformation control where not contaminated with other microorganism were not found and the cell competency were still good enough due to positive transformation result. Ligation control was made to confirm so the colony grows in ligation sample transformant were not false positive. All of the colony in plate A were picked into LB medium to isolate their plasmid. After isolation, dissolved plasmid was diluted with 20 times factor to set as template for confirmation procedure.

First confirmation would be a comparison of migration profile has a desired result that isolated plasmid were slower (have short migration) than pCAMBIA1303 (Figure 5). This should indicate that a isolated plasmid have weighs heavier because of inserted *dbr2* gene, but this result could not be as strong evidence. Next confirmation was restriction analysis between inserted vector with empty vector. As the result, a different pattern of



Figure 4. Transformation result of (-) = negative transformation control, (+) positive transformation control, B = ligation control, and A = ligation sample.

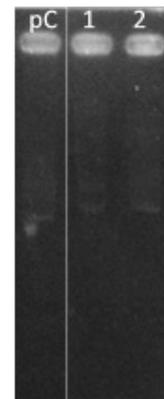


Figure 5. Electrophoregram of migration profile comparison between isolated plasmid 1 and 2 = colony 1 and 2, with pC = pCAMBIA1303.

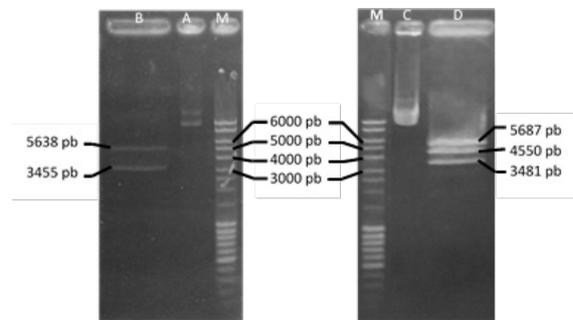
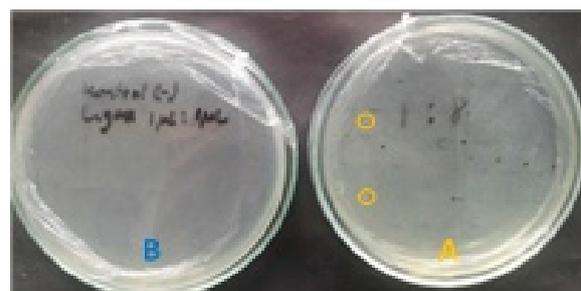


Figure 6. Electrophoregram of restriction analysis between D = isolated plasmid and B = pCAMBIA1303 relative to uncut plasmid C and A respectively.

fragment that indicates different size between them (Figure 6). In fact, the reason for using *NheI* and *SacI* enzyme provided the same number of fragments (theoretically three) but has different size. Although first and third fragment size were the same (around 5600 bp and 3400 bp), the second fragment size showed differences, for empty vector the fragment size was similar to the third fragment size (around 3300 bp) so that it looks like one fragment instead of two (Figure 6). It could be imagined from inserted vector simulation



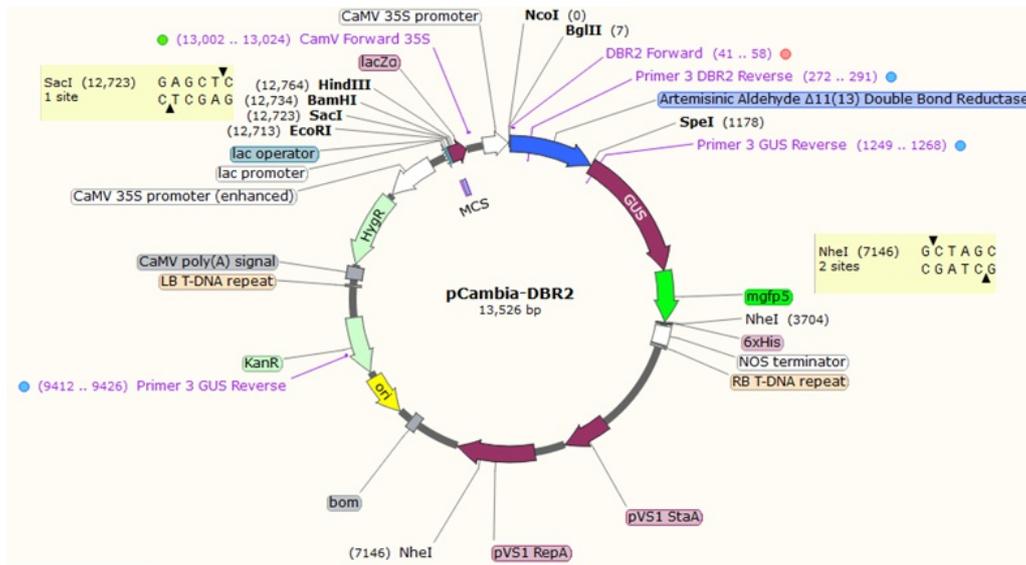


Figure 7. pCambia-*dbr2* map simulation with *NheI* and *SacI* restriction sites at *NOS* terminator, *pVS1 RepA*, and *MCS* are respectively.

(Figure 7) that *dbr2* insert gene make the fragment becomes longer. This analysis also could not strengthening the existing evidence because the addition of fragment size could be from any genetic material not specifically mention it was *dbr2* gene. Therefore, further confirmation analysis was needed.

Further confirmation was PCR analysis using *dbr2* specific primer with pUC57-*dbr2* as a positive control. Optimization of annealing temperature was needed to be done using gradient PCR to get the suitable PCR system. The right annealing temperature has bold and clear amplification band than others, therefore lane H was the right one (Figure 8), then *dbr2* specific system was set 46°C for annealing temperature. Isolated plasmid were confirmed using optimized PCR system. As the result, two band samples were linear with positive control at around 1110 bp (Figure 9),

it could be ascertained the plasmid samples have *dbr2* inserted gene. For the purpose of artemisinin biosynthetic engineering, how the nucleotide sequence and reading position was important, and the previous result was not strong enough to convince that plasmid system would be expressed well. To overcome these doubts, DNA sequencing analysis was performed using MacroGen’s service as a third party. Firstly, the samples was analyzed using two primers (*CaMV35S* Forward and primer 3 *DBR* Reverse) and the result was identical with Genebank database, then to complete ORF sequences, two other primers (*DBR* Forward and primer 3 *GUS* Reverse) were used. These four sequences result was combined using BioEdit program, then use nucleotide BLAST™ tools from NCBI to be compared with database. Identity value reached 100% score for *dbr2*, it means the

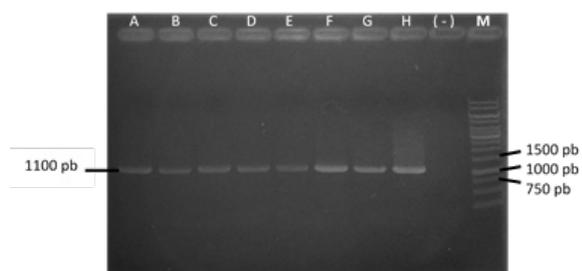


Figure 8. Electrophoregram of optimizing annealing temperature at A = 53,0°C, B = 52,4°C, C = 51,6°C, D = 50,4°C, E = 48,7°C, F = 47,3°C, G = 46,5°C, H = 46,0°C, with (-) = negative control.

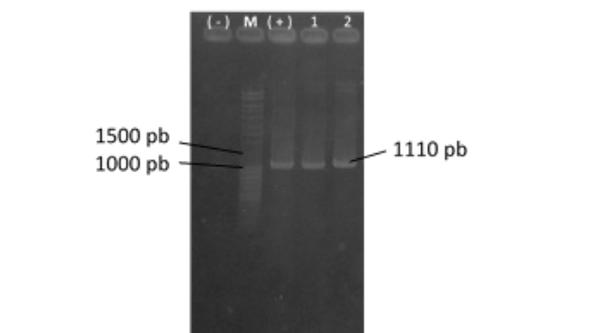


Figure 9. Electrophoregram of PCR analysis from 1 and 2 = isolated plasmid compared with DNA ladder (M).

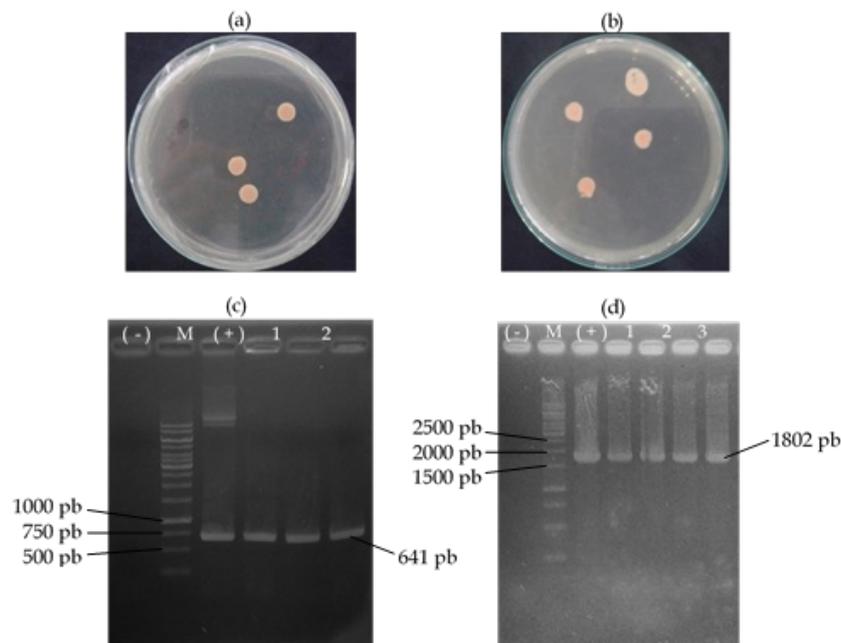


Figure 10. *A. tumefaciens* AGL1 transformant contains A = pCAMBIA1303, and B = pCAMBIA-*dbr2* as evidenced by electrophoregram of crude PCR C and D respectively.

whole sequence of *dbr2* was completely similar to sequence from library. Because of similarity with database, it should strongly concluded that gene construct was successfully done.

Confirmed pCAMBIA-*dbr2* and pCAMBIA1303 plasmid were transformed into *A. tumefaciens* AGL1 competent cell to get recombinant transformed colonies. To ensure that colonies contain desired gene, crude colony PCR was done with all of grown colonies on the plate medium (Figure 10). All of PCR product indicates all colonies were positive due to comparison with control plasmid. Next step was genetic transformation into *A. annua* which was mediated by *A. tumefaciens* AGL1 recombinant. This stage was performed using four groups of transformation: pCAMBIA-*dbr2*, pCAMBIA1303, AGL1 (wild type), and without transformation (plant wild type). The aim of this treatment was to observe the gene expression in short period of time, so called transient

transformation. Transient transformation was a preliminary test to determine level of transformation and expression result of transferred gene. The transformation was preceded by harvesting *A. tumefaciens* AGL1 cultures at $OD_{600} \approx 1$ to get a best result of transfection (Chamidah, 2013). Strain AGL1 has hypervirulent properties than others and made a tough transformation result using GUS histochemical evaluation (Elfahmi, 2014) so it was chosen. This study used a young leaves (near apical) explant to proceed *dbr2* gene transformation, because it has either minimum contaminant or minimum barrier that possible for *A. tumefaciens* infection and also in this part has amount of artemisinin than others.

Acetosyringone as a phenolic compound would trigger gene delivery system through signal transduction mechanism in *A. tumefaciens* cell surface. Silwet S-408 is organosilicon surfactant acts to lower surface tension between bacteria and plant cell, this

Table 6. Measurement area of blue spot of leaves using *ImageJ* software.

Treatment Group	Repetition			Mean Proportion of Blue Spot (%)	Coefficient of Variance (%)
	1	2	3		
pCAMBIA1303	94.63%	97.37%	94.89%	95.63%	1.29%
pCAMBIA- <i>dbr2</i>	94.31%	91.90%	91.97%	92.73%	1.21%
AGL1	0	0		0	0
TT	0	0		0	0

type of surfactant gave the best transformation result compared with other series (Chahyadi, 2011), concomitant use with acetosyringone could increase transformation efficiency. *Agrobacterium*-mediated gene transformation efficiency could be enhanced using vacuum infiltration method by creating negative atmospheric pressure conditions then the air in the space between plant cells reduced and facilitate the bacteria to have direct contact with the cells. After three days cocultivation, leaves explant were washed by cefotaxime to kill excessive bacteria attached to the leaves, and were divided for subsequent expression confirmation.

The blue spot on pCAMBIA-*dbp2* leaves transformant after GUS histochemical assay showed an expression of GUS reporter gene that linked to the *dbp2* gene, it means that two genes has been in-frame (Figure 11). Percentage of blue spot area in triplicate indicates level of gene expression area, the wider of blue spot area the better transformation efficiency Table 6). Staining time to get blue spot area for its triplicate were in 3 days, 5 days, and 4 days respectively. The difference of staining time was caused by the sensitivity of stored X-Gluc staining solution diminished over time. However, repeatability of transformation method could still be accepted due to CV (coefficient of variance) value was below 5% relatively.

To determine the existence of *dbp2* gene recombinant on pCAMBIA-*dbp2* leaves transformant, DNA genomic isolation was performed. It was used as a template for PCR reaction with primer of FCaMV35S and RGUS

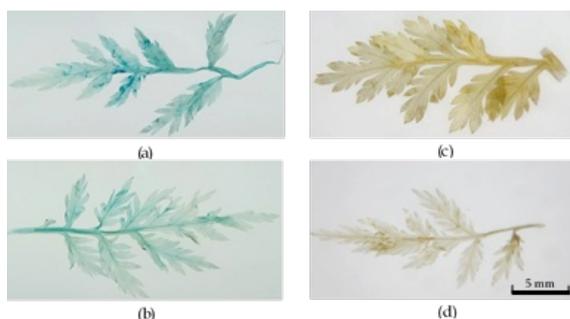


Figure 11. Leaves of GUS staining result for A = pCAMBIA1303, B = pCAMBIA-*dbp2*, C = AGL1, D = without transformation.

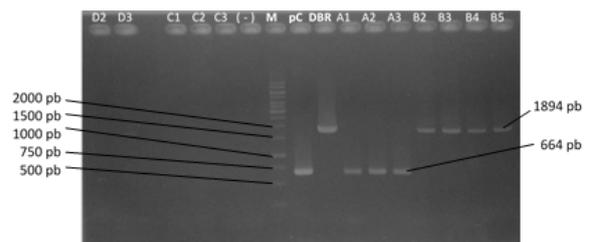


Figure 12. Electrophoregram of PCR product of isolated leaves genomic from transformant of A1-A3 = pCAMBIA1303, B2-B5 = pCAMBIA-*dbp2*, C1-C3 = AGL1, D2 and D3 = without transformation are compared with result of pC = pCAMBIA1303 plasmid, DBR = pCAMBIA-*dbp2* plasmid control

in spite of *dbp2* specific primer because it would give bias result with the existing *dbp2* native gene in wild type genomic DNA. There was PCR product band with 664 bp size at A1-A3 lane same as pCAMBIA1303 band control which size of CaMV35S to GUS primer region, yet product band at B2-B5 lane have 1894 bp size (Figure 12) means have extra around 1230 bp which is similar to inserted *dbp2* gene size.

Conclusions

Gene construction of artemisinic aldehyde $\Delta 11$ (13) double bond reductase (*dbp2*) into pCAMBIA1303 binary vector has been successfully preconcerted after through four steps confirmation test. GUS histochemical assay after pCAMBIA-*dbp2* transient transformation provide the blue spot area which means *dbp2* gene has been transformed as was in-frame to GUS reporter gene and its existence was proved by PCR of genomic DNA. Verification of dihydroartemisinic aldehyde and artemisinin content is needed between non-recombinant compared to recombinant transformant.

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