JJBS

Volume 17, Number 2,June 2024
ISSN 1995-6673
Pages 235 – 246
https://doi.org/10.54319/jjbs/170203

Jordan Journal of Biological Sciences

Overexpression of 3-hydroxy-3-methylglutaryl coenzyme-A reductase-1 *(HMGR1)* enhanced andrographolide accumulation in *Andrographis paniculata* (Burm.F.)

Erly Marwani[1,*] , Juane Plantika Menra , Amanda Nadhira , Tati Kristianti[2], Karlina Febrianti , Fenny Martha Dwivany

[1]*School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung 40132, Indonesia;*[2] *Institut Pendidikan Indonesia, Garut West Java, Indonesia*

Received: June 1, 2023; Revised: August 15, 2023; Accepted: September 18, 2023

Abstract

3-hydroxy-3-methylglutaryl coenzyme-A reductase (*HMGR*) enzyme plays an important role in the biosynthesis of diterpene lactones such as andrographolide, a specialized metabolite that is widely used as a therapeutic agent. This study aimed to enhance the content of andrographolide through the overexpression of 3-hydroxy-3-methylglutaryl coenzyme-A reductase-1 (*HMGR1*) in *A. paniculata*. *HMGR1* was constructed into the pBI121 expression vector controlled by the cauliflower mosaic virus (CaMV 35S) promoter. The pBI121-*HMGR1* construct was transformed to *A. paniculata* through Agrobacterium-mediated transformation followed by plant regeneration to obtain transgenic plants. Polymerase chain reaction (PCR) analysis was conducted to validate the transgenic plants, quantitative reverse transcription PCR (RT-qPCR) was used for the *HMGR1* expression analysis, and high-performance liquid chromatography was employed to quantify the andrographolide content of the transgenic plants. PCR analysis on the transgenic plantlets indicated the presence of a 2,262 bp fragment that corresponded to *HMGR1*, thus confirming the integration of *HMGR1* into the *A. paniculata* genome. The RT-qPCR results showed a 52.5-fold greater *HMGR1* expression in the transgenic plant under the CaMV 35S promoter compared to the normal plant. As the expression level of *HMGR1* increased, the amount of andrographolide was enhanced by up to 2.6 times in the transgenic plant compared to the control plant. This study demonstrates that the overexpression of *HMGR1* driven by CaMV 35S can enhance the production of andrographolide in *A. paniculata*.

Keywords: Andrographolide, overexpression, transgenic, HMGR, CaMV 35S .

1. Introduction

Andrographis paniculata has long been recognized as a traditional medicinal plant in various tropical and subtropical countries, including Thailand, Indonesia, Malaysia, Sri Lanka, and India (Akbar, 2011; Benoy *et al*., 2012; Kabir *et al*., 2014). Extract from the plant has shown a broad spectrum of pharmacological activities, i.e. immunostimulatory (Kumar *et al*., 2004), antiviral/anti-HIV (Churiyahi *et al*., 2015), antibacterial (Jarukamjorn and Nemoto, 2008), anticancer (Harjotaruno *et al*., 2008), antidiabetic, antimalarial, hepatoprotective (Jarukamjorn and Nemoto, 2008), and anti-inflammatory (Chandrasekaran *et al.,* 2012) activities. Currently, the dried extract of *A. paniculata* in capsule or dried leaf form is available commercially and is used as an immunostimulant.

Based on the identification of its chemical constituents, *A. paniculata* contains various bioactive compounds in the form of diterpenoids, flavonoids, and polyphenols (Chao and Lin, 2010). Among the diterpenoid group in *A. paniculata*, andrographolide has previously been identified as the main bioactive compound (Rao *et al.,* 2004). Andrographolide is a bitter crystalline compound, colorless, with various biological activities, namely anti-HIV, as shown by its inhibition of the HIV-induced cell cycle dysregulation (Calabrese *et al.* 2000), antiviral, as demonstrated by its viricidal activity against the herpes simplex virus (Wiart *et al.*, 2005), as well as antipyretic, immunostimulant, analgesic, anticancer (Suebsana *et al.,* 2009), and anti-inflammatory activities (Levita *et al.,* 2010). Recently, *A. paniculata* extract and andrographolide compounds have been reported to potentially show anti-SARS-CoV-2 activity (Shi *et al*., 2020), Additionally, based on the results of in silico studies, it has been hypothesized that andrographolide may show anti-SARS-CoV-2 activity (Lakshmi *et al.*, 2021; Enmozhi *et al*., 2020; Laksmiani *et al*., 2020). However, andrographolide has only been detected in the leaves of *A. paniculata* at a concentration of 2.2% (Royani *et al.,* 2014). As the market need for andrographolide grows, so does the need to develop a technique for improving the andrographolide levels in *A. paniculata*. Metabolic engineering is one of the promising techniques used to enhance secondary metabolite accumulation.

Metabolic engineering to increase secondary metabolites can be performed in different ways. These

* Corresponding author. e-mail: erly@itb.ac.id.

include the overexpression of certain genes to regulate metabolic pathways (Alvarez and Marconi, 2011). Similarly, Lu *et al.* (2016) stated that overexpressing genes in the terpenoid biosynthetic pathway in homologous and ectopic plants provided a reliable method for increasing terpenoids. In addition, overexpression of the *PGSQS1* gene in the ginsenosides biosynthesis pathway may enhance the expression of β-amyrin synthase (β-AS) and cycloartenol synthase (CAS), resulting in increases in phytosterols and ginsenosides of up to 2.0 and 1.6 times, respectively, in root cultures of transgenic ginseng (Shim *et al.*, 2010). Moreover, Han *et al.* (2013) reported an increase in oleanane-type ginsenoside in *P. ginseng* plants following overexpression of the *CYP716A52V2* gene. Similar results have shown that overexpression of the 3-hydroxy-3-methylglutaryl coenzyme-A reductase (*HMGR*) gene produced a 1.5- to 2-fold rise in the ginsenosides content of adventitious ginseng root cultures resulting from genetic transformation (Kim *et al.*, 2014).

Recently, overexpression of the *HMGR* gene in transgenic roots and stems of *Ligularia fischeri* has been shown to increase the shionone content by up to 16.67% and 12.25%, respectively, when compared to controls (Juan Du *et al.*, 2020). When seeking to overexpress a gene to enhance enzyme activity, the proper promoters should be selected. The cauliflower mosaic virus (CaMV 35S) promoter is frequently used as a standard promoter for transgene expression in plants (Somssich, 2019; Amack and Antunes, 2020). It is known as a strong, constitutive promoter and is frequently used in expression in transgenic plants (Somssich, 2019).

Song *et al.* (2012) reported how the use of the CaMV 35S promoter for overexpression of the endogenous *HMGR* gene enhanced the accumulation of β-sesquiphellandrene by 1.25–1.60 fold. Similarly, Dong *et al.* (2013) reported that overexpression of one of the isoprenoid pathway enzymes, i.e. *HMGR*, in *Parthenium argentatum* increased carbon flux to the isoprenoid pathway; for example, for the synthesis of plant growth substances. Moreover, Putter *et al.* (2017) indicated that overexpression of the three main enzymes in the mevalonate cascade, which include *HMGR*, in *Taraxacum brevicorniculatum* can increase enzyme activity and result in an increase in high squalene concentrations in dandelion roots. Another report indicated that 3-hydroxy-3-methylglutaryl-CoA reductase-1 (*HMGR1*) showed a correlation with andrographolide biosynthesis in *A. paniculata* (Jha *et al*., 2011).

Our interest in this study was to employ metabolic engineering in a bid to enhance the amount of andrographolide through overexpression of the *HMGR1* gene involved in the mevalonate pathway, a biosynthetic pathway that produces various secondary metabolites including diterpenoids such as andrographolide. To date, however, no overexpression of the *HMGR1* gene driven by CaMV 35S in *A. paniculata* to enhance the accumulation of andrographolide has been reported. Therefore, the present research aimed to overexpress the *HMGR1* gene in *A. paniculata* under the control of a strong promoter, namely CaMV 35S. This was expected to lead to an increase in the accumulation of andrographolide. This paper reports the construction of the *HMGR1* gene into pBI121 plasmid, the transformation of *A. tumefaciens* with this recombinant plasmid, the development of an *A. paniculata* transgenic line using *Agrobacterium* transformation method, plant regeneration, gene expression, and andrographolide analysis of the *A. paniculata* transgenic plant.

2. Materials and Methods

2.1. Plant Material

A. paniculata seeds and mature plants were obtained from the Research Institute for Medicinal Plants and Spices (Balitro), Bogor, West Java. The plant material used for the isolation of RNA comprised three-month-old leaves from the *A. paniculata* plant after Sharma *et al.* (2013) reported that the leaves of *A. paniculata* contain higher levels of andrographolide (2–3%) compared to other parts of the plant. Meanwhile, the explants used for genetic transformation were cotyledons of three-week-old *A. paniculata* seedlings**.**

2.2. Bacterial Strain and Plasmid

pGEM-T Easy from Promega was employed as the cloning vector to amplify the *HMGR1* gene. *Escherichia coli* strain DH5α was used in the cloning of pGEM-T Easy-*HMGR1*, while pBI121 was used as the binary vector for construction. *Agrobacterium tumefaciens* strain GV3101 was used for plant transformation, which was provided by Dr. Sony Suhandono from the Molecular Genetics laboratory, School of Life Sciences and Technology, Institut Teknologi Bandung.

2.3. Medium and Reagents

The medium for plant regeneration was prepared according to the protocol from Marwani *et al.* (2013). GoTaq® Green Master Mix was the reagent used for Polymerase Chain Reaction (PCR), which was obtained from Promega™. Agarose (Top Vision Agarose), and 1 Kb ladder were used for gel electrophoresis and were obtained from Fermentas™. The purification kit for gel electrophoresis was obtained from Geneaid™. The primers were sourced from Macrogen Inc.

2.4. Amplification of HMGR1 cDNA

The *HMGR1* cDNA was first isolated from *A. paniculata* leaf by Menra (2013). The *HMGR1* cDNA was amplified using touchdown PCR (Applied Biosystems 2720 thermal cycler) with specific primers for *HMGR1*, forward primer 5'AGA ATG GCC GCC CTC CG3' and reverse primer 5'ATC GAC TCT CTC TGT CTC CAA TCT CAA GT3', to give a DNA length of around 1,800 bp. The PCR reaction comprised 25 µl GoTaq® Green Master Mix, 2.5 µl *HMGR1* forward primer, 2.5 µl *HMGR1* reverse primer, 1 µl cDNA template, and 19 µl nuclease-free water. The process began with initial denaturation for 3 minutes at 95°C followed by 10 cycles of amplification. Annealing and extension were performed at 50–60^{o}C for 30 seconds and 72°C for 2.5 minutes, respectively. The PCR products were subjected to electrophoresis, followed by visualization using ethidium bromide staining, and observed under UV light irradiation for later documentation. The *HMGR1* fragment was then purified using a gel purification kit from Geneaid in line with the procedure provided by the kit manufacturer. Next, the fragments from the purification results were ligated into the pGEM-T Easy vector from Promega. The ligated

pGEM-T-Easy-*HMGR1* was subsequently used to transform competent *E. coli* DH5α cells using heat shock transformation following the method used by Chang *et al.* (2017). The transformants were spread onto LB agar containing ampicillin (100 mg/l), IPTG (0.1 M), and X-gal (100 mg/l), and subsequently incubated at 37°C for 16 hours to select the recombinant clones. White colonies indicated the presence of pGEM-T-Easy with the *HMGR1* gene inserted.

A single colony of transformants was selected and suspended in a 5 ml LB medium containing 100 ppm of ampicillin and stored in the shaker at 37°C, 200 rpm overnight. The plasmids were isolated according to the alkaline lysis method employed by Amara (2018), with modification. The isolated plasmid of *HMGR1* gene-pGEM-T-Easy was sequenced at Macrogen Inc. (Seoul Korea) to test for the presence of the *HMGR1* gene using primers T7 and SP6. The sequencing results were then analyzed by bioinformatics using BLAST software at the NCBI website (http://ncbi.nlm.nih.gov/blast).

2.5. Construction of pBI121-HMGR1 Plasmid Vector

pBI121 was used as the backbone in constructing the expression vector carrying the *HMGR1* gene. The *HMGR1* gene would be expressed under the CaMV35S promoter. The multiple cloning sites at the pBI121 plasmid contained *Xba*I, *Bam*HI, *Sma*I, *Xma*I, and *Bsr*GI restriction sites between the CaMV 35S promoter and *GUS* gene. Of the five restriction enzyme recognition sites, *Xba*I and *Bsr*GI do not cleave the ***HMGR1*** gene. Therefore, a pair of primers was designed with *Xba*I (5-TCT AGA AGA ATG GCC GCC CTC CGC CGC CGA) and *Bsr*GI (TGT ACA AAT CGA CTC TCT CTG TCT CTC AAT) recognition sequences added to the forward and reverse primers, respectively. These were then used to amplify the ***HMGR1*** gene in the recombinant pGEM-T Easy. Next, the amplicon was ligated into the pBI121 fragment digested with *Xba*I and *Bsr*GI, with a volume ratio of 3:1 using T4 DNA ligase. The ligation reaction comprised 3 µl DNA insert, 1 µl DNA backbone, 1 µl *Xba*I enzyme, 1 µl T4 DNA ligase, 1 µl T4 DNA Ligase buffer, and 13 µl deionized water. The reaction was performed at 4°C for 16–18 hours. Ligation produced a recombinant pBI121-*HMGR1* construct with a length of 15,004 bp (Figure 1).

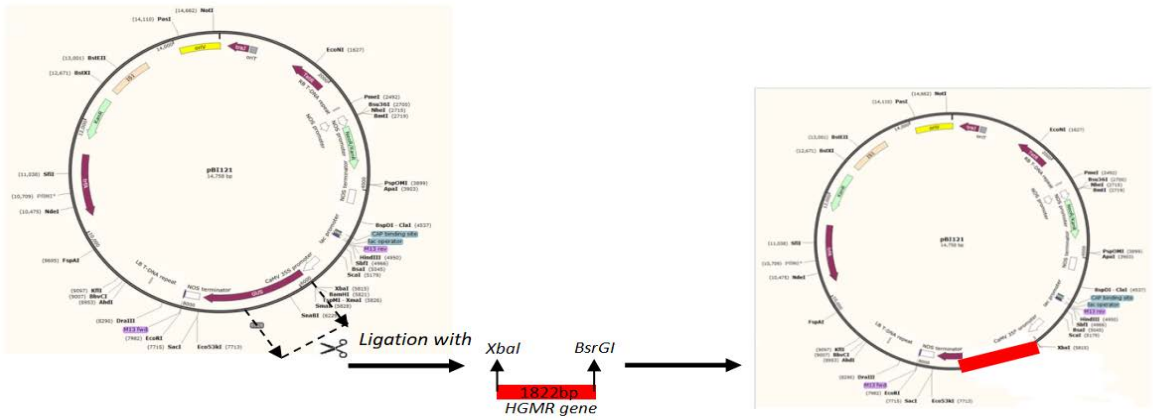

Figure 1. Diagram of the construction of *HMGR1* in binary vector pBI121

Applying the heat shock transformation method of Chang et al. (2017), the pBI121-*HMGR1* construct was transformed into competent cells of E. coli DH5α. Afterward, the E. coli DH5α containing the pBI121-*HMGR1* construct was cultured in LB medium supplemented with 50 mg/l kanamycin at 37°C. The plasmid was subsequently isolated using the alkaline lysis method according to Amara's (2018) standard protocol with modification. To confirm the success of the plasmid ligation, the isolated pBI121-HMGR1 fragment was digested with *Xba*I and *Bsr*GI restriction enzymes and observed by 1% agarose gel electrophoresis. The restriction digest resulted in two fragments with sizes of 1,822 bp and 13,182 bp. The final step was sequencing the *HMGR1* gene in pBI121-*HMGR1* recombinant plasmid at Macrogen Inc., Seoul, Korea. Bioinformatics analysis was performed on the sequencing results using the BLAST nucleotide program from NCBI (www.ncbi.nlm.nih.gov).

2.6. Transformation of A. tumefaciens *GV3101 with Recombinant Plasmid pBI121-HMGR1*

Agrobacterium tumefaciens strain GV3101 was transformed with the constructed pBI121-*HMGR1* plasmid using heat shock based on the procedure of Chang *et al.* (2017) with a slight adjustment. A total of 2–5 µl pBI121-*HMGR1* plasmid was placed in 50 µl competent *A. tumefaciens* cells in microtubes. Afterward, the tubes were inverted repeatedly and then incubated in ice for 10 minutes or immersed in liquid nitrogen for 5 minutes. Next, the microtubes were incubated in a water bath at 37°C for 25 minutes and 1 ml of liquid YEP (10 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, 15% bacto agar, pH 7) medium consisting of 50 mg/l kanamycin and 50 mg/l rifampicin was added and grown at 25°C, 200 rpm in the dark for 3 hours and then centrifuged at 14,000 rpm for 1 minute. The supernatant was removed, and the pellet was suspended and plated on a solid YEP medium consisting of 50 mg/l rifampicin and 50 mg/l kanamycin and stored in an incubator at 25°C for 2 days. A selected colony was suspended in 25 µl deionized water, and 3 µl of the

suspension was applied to the PCR assay to test for the presence of the *HMGR1* gene.

The PCR mixture comprised 5 µl GoTaq® Green Master Mix, 1 µl forward primer, 1 µl reverse primer, 1 µl DNA template, and deionized water to a total volume of 10 µl. PCR was performed using specific primers for the *HMGR1* gene (forward 5'-TCT AGA AGA ATG GCC GCC CTC CGC CGC CGA-3' and reverse 5'-TGT ACA AAT CGA CTC TCT CTG TCT CTC AAT-3'), giving an expected DNA length of around 1,822 bp. Amplification was set as follows: initial denaturation at 95°C for 3.5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 2 minutes, and final elongation at 72°C for 7 minutes. The PCR product was analyzed on 1% agarose gel electrophoresis (Top vision agarose) and 1 Kb DNA Ladder #331 (Fermentas). A DNA band of 1,822 bp indicated the presence of the *HMGR1* gene.

2.7. Transformation and Regeneration of Andrographis paniculata

2.7.1. Preparation of A. tumefaciens

A colony of *A. tumefaciens* GV 3101 containing recombinant plasmid pBI121-*HMGR1* was suspended in 25 ml yeast extract peptone medium enriched by 50 mg/l kanamycin and 25 mg/l rifampicin and incubated overnight at 28°C, 200 rpm in the dark. The *Agrobacterium* cells were collected after centrifugation at 4000 rpm for 20 minutes. The pellet of *Agrobacterium* cells was re-suspended in liquid half-strength Murashige and Skoog (MS) medium to reach a concentration of 10^6 cells/ml or OD_{600nm} = 0.8. Finally, 100 µM acetosyringone and Silwet L-77 with a ratio of 1:3 was added into the *A. tumefaciens* suspension.

2.7.2. Agrobacterium-mediated Genetic Transformation, Co-cultivation, and Regeneration

For the purpose of plant transformation, the *A. tumefaciens* GV3101 harboring pBI121-*HMGR1* was used to infect *A. paniculata* by agroinfiltration. First, the cotyledons of *A. paniculata* were immersed for 60 minutes in the *A. tumefaciens* suspension. Subsequently the cotyledons were drained on sterile filter paper and co-cultivated for 3 days on agar-solidified (0.8% w/v) MS medium enriched with 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l indole-3-acetic acid (IAA), 1% Silwet L-77, 100 µM acetosyringone, and 50 mg/l kanamycin at 28°C in the dark. The tissue was then rinsed using sterilized distilled water 3 times for 5 minutes and immersed for 15 minutes in cefotaxime 400 mg/l or augmentin 300 mg/l to eliminate bacteria. The cotyledons were then transferred to an MS medium containing 2 mg/l BAP, 1 mg/l IAA, 250 mg/l augmentin, and 350 mg/l cefotaxime for 6 days according to the method of Konagaya *et al.* (2020). The surviving tissues were transferred to a selection medium (solid MS medium consisting of 2 mg/l BAP, 1 mg/l IAA, kanamycin 20 mg/l) at 25°C for 2 weeks under a 36-watt Tungsten lamp (TL) with a light intensity of 1000 lux and 12-hour photoperiod. To induce shoot formation, the transformed cotyledon tissues were moved to a shoot regeneration medium (solid MS medium consisting of 2 mg/l BAP, 1 mg/l IAA with the addition of 20 mg/l kanamycin) and incubated at 25°C for 2 to 3 weeks under a 12-hour photoperiod. To induce root formation, the regenerated shoots were implanted on a solid MS medium with the addition of 1 mg/l indole butyric acid (IBA) and incubated at 25°C for 3 to 4 weeks until the primary plantlets were formed.

Transformed plantlets (transgenic progeny) that measured 7–10 cm in length and were vigorous were carefully removed from the culture bottle, cleaned under running tap water to remove adhered agar, and subsequently planted in a plastic pot containing a mixture of sterilized husks and sandy soil with a 1:1 ratio and maintained in a greenhouse at 27°C under a 12-hour photoperiod. To prevent excessive evaporation, the plastic pots were covered with transparent plastic for the first month, which was subsequently removed when the plants showed more vigor. The transgenic plants were cultivated in the pots for 3 months, after which the leaves, stem, and roots were harvested to determine the andrographolide concentration.

2.8. PCR of Transgenic Plant

PCR was performed to test the integration of the *HMGR1* gene into the *transformed A. paniculata* genomic DNA following the method of Han *et al*. (2017) with a slight modification. The genomic DNA of three biological replicates (marked as TA1, TA2, and TA3) of the T0 generation transformed plantlets were isolated using the Plant Genomic DNA Kit from Tiagen. Up to 1 µl of the isolated DNA was mixed with 5 µl GoTaq® Green Master Mix, 1 µl forward primer, 1 µl reverse primer, and 1 µl deionized water. PCR analysis was performed using specific primers of pBI121 (forward 5'-TTC GTC AAC ATG GTG GAG CA and *HMGR1* reverse 5'-TGT ACA AAT CGA CTC TCT CTG TCT CTC AAT-3'), resulting in a DNA length of around 2,262 bp. The PCR cycle was programmed as follows: initial denaturation at 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes, and finally, elongation at 72°C for 7 minutes. The PCR product was applied to 1% agarose gel electrophoresis and observed under a UV transilluminator.

2.9. RNA Isolation and Quantitative Real-Time Expression Analysis of HMGR1 Gene in Transformed A. paniculata

Using the TRIzol (Thermo Fisher Scientific, United States) procedure, transformed *A. paniculata* RNA was isolated from three transformed plant tissues, labeled TA1, TA2, and TA3, as well as from the control plant tissue. Trizol was used in this work because, compared to other techniques, it has a better reputation for isolating RNA from plant tissues (Mohammed *et al*., 2019). The quality of the RNA was assessed based on concentration and electrophoretic bands. RNA with an $A_{260}/A_{280} \geq 1.8$ was selected due to its high purity, as stated by Sukumaran (2011). The total RNA was converted into cDNA using a Sensi FAST cDNA Synthesis Kit from Bioline. The cDNA reaction (20 µl total volume) consisted of 8 µl total RNA (100 ng), 4 µl TransAmp Buffer, 1 µl Reverse Transcriptase, and 7 µl nuclease-free water. The following program was established in a thermal cycler: 25°C for 10 min (primer annealing); 42°C for 15 min (reverse transcription); 85°C for 5 min (inactivation), and 4°C hold (or chill on ice). In the RT-qPCR assays, each reaction (20 µl total volume) consisted of 2 µl diluted cDNA, 10 µL qPCR Promega master mix, 1 µl each forward and reverse

primers, and 6 µl RNAse-free water. To ensure reliability and validity, three technical replicates were performed per sample. The RT-q PCR cycle was programmed as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, and 56°C for 30 s. The specific primers were determined using Primer 3 software (Table 1). The relative expression levels of the genes were normalized against the actin housekeeping gene and were later expressed as a function of the non-transgenic (control) plant values, whose *HMGR1* transcript levels were defined as one (1) in line with the method of Livak and Schmittgen (2001). The relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where ΔCt= Ct target -Ct reference and ΔΔCt= ΔCt transgenic plant - ΔCt control (non-transgenic plant). An average of three biological replicates (three transformed tissues) were used to calculate the expression levels.

Table 1. List of primers for relative expression analysis

Primers	Sequences	Product size
Actin-F	5'GAGGTGCTCTTTCAGCCATC'3	250bp
Actin-R	5'TTGATCTTCATGGTGCTTGG'3	
HMGR-F	5'GCATCGGTTTTGTCCAGTCT'3	250bp
HMGR-R	5'GAAGAGGCGATTTTGCAGTC'3	

2.10. Determination of Andrographolide Content

Analysis of the andrographolide content in transgenic and non-transgenic *A. paniculata* was conducted using high-performance liquid chromatography (HPLC) Shimadzu CR-and A Plus according to the method of Majee *et al.* (2011) with modification. Leaf, stem, and root samples from the transformed and non-transformed (control) A. *paniculata* were harvested from the age of 3 months. Triplicate samples of leaf, stem, and root were used for the HPLC analysis of andrographolide. The samples were freeze-dried for 24 hours followed by extraction with methanol (sample: methanol ratio of 1:10) for 48 hours. The crude extracts were filtered using a polytetrafluoroethylene (PTFE) membrane, after which the solvent was evaporated using a vacuum evaporator. The dried extracts were dissolved in 1 ml HPLC grade methanol. The extracts were then loaded into an HPLC system equipped with a UV detector, Bondapak C_{18} column (Ø 4.6 mm x 250 mm), using methanol: H_2O (6:4) as the mobile phase at a flow rate of 1 ml/min, and monitored at λ 254 nm. The reference solution was the andrographolide compound purchased from Sigma-Aldrich. To produce the calibration curve for andrographolide, a standard andrographolide solution was prepared in a separate flask using an appropriate dilution of 0.5 mg/ml stock solution with the same volume of solvent (HPLC grade methanol) to final concentrations of 0.25, 0.125, 0.0625, and 0.0312 mg/ml. Each standard solution was injected into HPLC, and each peak area value was plotted to the corresponding concentration to obtain the linear calibration curve. A linear equation was obtained from the linear curve, and the concentration of the injected samples was determined.

2.11. Statistical analysis

Statistical analysis was conducted to assess the significance of the difference in the data variation for the andrographolide concentrations and the number of shoot regenerations in the normal and transformed tissues. The analysis was performed using SPSS Statistics software version 29, employing a one-way ANOVA (analysis of variance) test. A p-value of less than 0.05 was considered to indicate statistical significance. The ANOVA test was followed by a post-hoc Tukey test to observe the variations in andrographolide concentration between the transformed and normal tissues. The andrographolide concentration data were presented as the mean ± SD (standard deviation) of triplicate biological samples.

3. Results

3.1. Construction of pBI121-HMGR1 Plasmid Vector

The cDNA fragment (around 1,800 bp) isolated from *A. paniculata* was verified as *HMGR1* by DNA sequencing (GenBank accession nos. AY429658.1). The insertion of the *Xba*I and *Bsr*G1 restriction enzyme site to the *HMGR1* fragment produced a fragment measuring between 1,500 bp and 2,000 bp in length (Figure 2A), which corresponded to the expected *Xba*I-*HMGR1*- *Bsr*G1gene (1,822 bp). The digestion of pBI121 with *Xba*I and *Bsr*GI restriction enzymes at specific nucleotide sequences eliminated the *GUS* gene and produced the pBI121 backbone with a length of 13,182 bp (Figure 2B), whereas digesting the pGEM-TEasy *HMGR1* with the same restriction enzymes produced a DNA fragment between 1,500 bp and 2,000 bp in length, which corresponded to the *HMGR1* gene (Figure 2C).

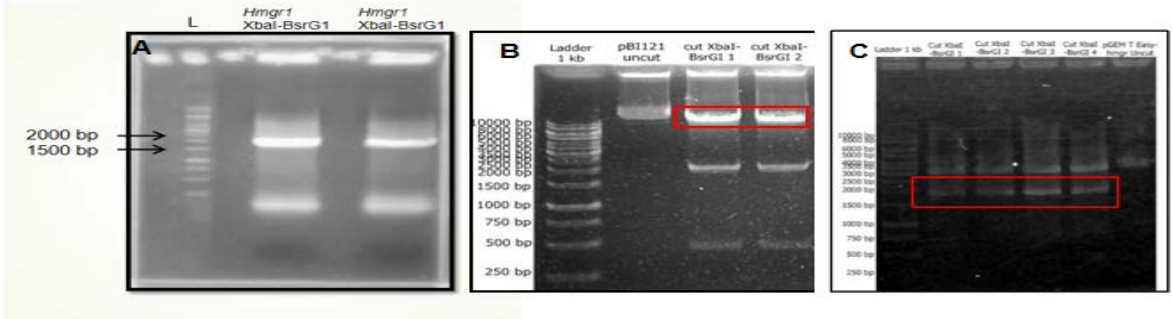

Figure 2. (A) *HMGR1* gene fragment containing *Xba*I and *Bsr*G1 restriction sites with a length of 1,500 bp–2,000 bp, corresponding to the size of *HMGR1* (1,822 bp). (B) Gel electrophoresis of pBI121 digested with *Xba*I and *Bsr*GI restriction enzymes, which produced the pBI121 backbone with a length of 13,182 bp, indicated by the red box. (C) Gel electrophoresis of pGEM-T Easy-*HMGR1* digested with *Xba*I and *Bsr*GI restriction enzymes, which produced a fragment with a length of 1,500 bp–2,000 bp, corresponding to the size of *HMGR1* (1,822 bp), indicated by the red box.

In general, restriction enzymes digest DNA at specific nucleotide sequences by hydrolyzing phosphodiester bonds on both DNA strands, producing a phosphate group at the 5' end and an OH group at the 3' end of the DNA strands (Allison, 2007), resulting in either cohesive or blunt ends (Koerniati and Widhianata, 2012). After purification by agarose gel electrophoresis, the pBI121 backbone and *HMGR1* insert were ligated using T4 DNA ligase, which produced a recombinant plasmid named pBI121-*HMGR1* with a length of 15,004 bp (Figure 3A). To test for the presence of *HMGR1* insert in the pBI121 plasmid, the recombinant pBI121-*HMGR1* plasmid was digested with restriction enzymes *Xba*I and *Bsr*GI. This produced two fragments, a 13,182 bp fragment corresponding to the pBI121 backbone and a fragment with a length between 1500 and 2000 bp (1,822 bp), which correspond to the *HMGR1* insert (Figure 3B).

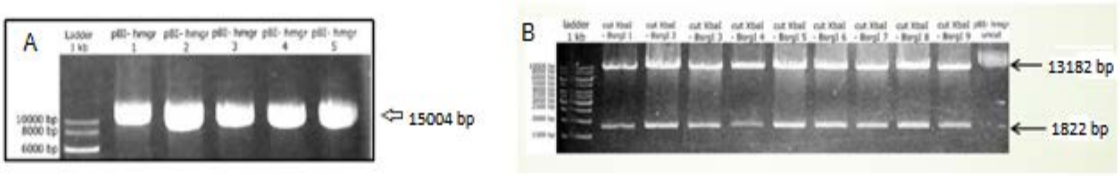

Figure 3. (A) The pBI121-*HMGR1* recombinant with a length of 15,004 bp. (B) Gel electrophoresis of pBI121-*HMGR1* plasmid digested with *Xba*I and *Bsr*GI restriction enzymes resulting in two bands.

As previously mentioned, the pBI121 backbone and HMGR1 insert were successfully ligated using T4 DNA ligase; this produced the recombinant plasmid pBI121-*HMGR1* with a length of 15,004 bp. To confirm this finding, the pBI121-*HMGR1* recombinant plasmid using *HMGR1* forward and reverse primers was sequenced and analyzed using the BLAST program at NCBI followed by alignment using BioEdit Sequence Alignment Editor version 7.2.5. High homology was exhibited between the pBI121-*HMGR1* sequence and *HMGR1 A. paniculata* from the NCBI database. The results indicated 99% identity and an expectation (E) value of 0.0 (Figure 4).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Andrographis paniculata 3-hydroxy-3-methylglutaryl-coenzyme A reductase (hmgr1) gene, complete cds	1670	1670	99%	0.0	99%	AY429658.1
Andrographis paniculata 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR1) mRNA, complete cds	1670	1670	99%	0.0	99%	AF389879.2

Figure 4. BLAST analysis of pGEM-T Easy *HMGR1*

3.2. Transformation of A. tumefaciens with pBI121-HMGR1 Plasmid

The successful transformation of *A. tumefaciens* with recombinant plasmid pB121-*HMGR1* was demonstrated by the ability of *A. tumefaciens* colonies to grow on the selection medium, i.e. medium with the addition of 50 mg/l kanamycin (Figure 5A). The non-transformed *A. tumefaciens*, in contrast, was unable to grow on that medium. This was caused by the kanamycin antibiotic's method of action, which prevents translation by disrupting ribosome functionality (Allison, 2007).

Moreover, a single band between 1,500 bp and 2,000 bp in length was detected via PCR amplification of *A. tumefaciens* cultured on a YEP medium containing kanamycin using a pair of specialized HMGR1 primers (Figure 5B). This result aligned with the *HMGR1* DNA fragment length of around 1,822 bp. This result demonstrated that the *A. tumefaciens* colony was successfully transformed and thus contained the pBI121-*HMGR1* construct.

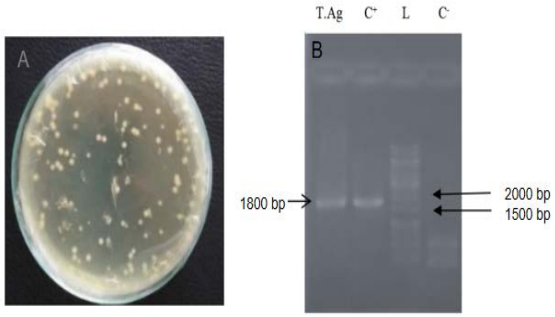

Figure 5. (A) Colonies of *A. tumefaciens* harboring recombinant plasmid pB121-*HMGR1* on YEP solid medium consisting of 50 mg/l kanamycin and 50 mg/l rifampicin. (B) Electropherogram of *A. tumefaciens* colony PCR product using a pair of *HMGR1 primers* (T. Ag: the colony of *A. tumefaciens*, C+: recombinant pBI121-*HMGR1*, L: Ladder 1 Kb, C-: negative control).

3.3. Transformation and Plant Regeneration

After transformation, some of the transformed cotyledonary tissues survived on the selection medium , MS medium consisting of 2 mg/l BAP, 1 mg/l IAA, and 20 mg/l kanamycin, (Figure 6A). However, the untransformed tissues did not grow on that medium and eventually died (Figure 6B). In the MS medium comprising 2 mg/l BAP and 1 mg/l IAA without kanamycin (control medium), the cotyledonary tissues grew well (Figure 6C). On days 28–30 following initiation, multiple new shoots (1–3 transgenic shoots for each explant) appeared directly from the transformed tissue when they were cultivated in the shoot regeneration medium, i.e. MS medium consisting of

2 mg/l BAP and 1 mg/l IAA, with the addition of 20 mg/l kanamycin (Figure 6D). Multiple shoots (7–9 shoots for each explant) only appeared from the non-transformed cotyledonary tissues when these tissues were cultivated in a medium with no kanamycin added (Figure 6E). In this medium, the non-transformed cotyledonary tissues produced multiple shoots more rapidly, i.e. 14–16 days after initiation. Thus, among the cotyledon explants, an average of 1.3 shoots per explant regenerated from the transformed tissue on the media with the addition of kanamycin. Meanwhile, an average of 8.0 shoots per explant regenerated from the non-transformed tissue on the control medium. The number of regenerated shoots from normal tissue was significantly higher ($P<0.05$) compared to the transformed tissue.

Roots were successfully formed in the transformed shoots (Figure 6F) on MS medium supplemented with 1 mg/l IBA and 20 mg/l kanamycin. Root formation also occurred in the non-transformed shoots on the same medium with no addition of kanamycin (Figure 6G). In this report, plant regeneration from transformed tissues was induced by direct organogenesis through direct shoot formation from the cotyledon explants followed by root formation to produce transgenic plantlets. Three of the transgenic plantlets were then transferred to a mixture of husk and soil (1:1) for acclimatization and maintained for further growth. The plantlets grew well in the plastic pots, and thus the three transgenic plantlets (TA1, TA2, and TA3) were further analyzed by PCR. Within 12 weeks of observation, the normal plants had grown higher than the transgenic plants. The non-transgenic (normal) plants reached 25 cm in height whereas the transgenic plants reached only 18 cm in height. However, no morphological differences were found between the transgenic and non-transgenic plants (Figure 6H).

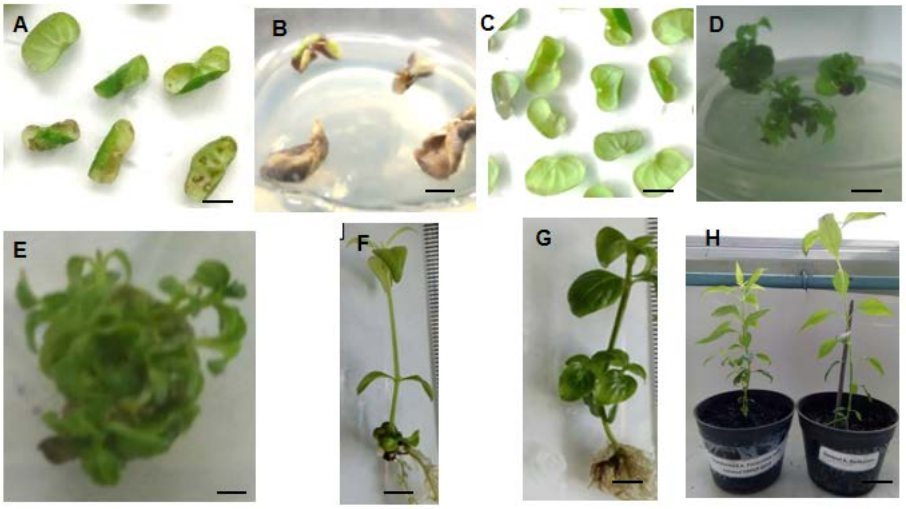

Figure 6. *In vitro* regeneration and acclimatization of plantlets from transformed cotyledon tissues of *A. paniculata* mediated by *A. tumefaciens* harboring recombinant plasmid pBI121-*HMGR1*. (A) Transformed cotyledonary tissues and (B) Non-transformed cotyledonary tissues on medium with the addition of kanamycin, (C) Cotyledonary tissues on medium without kanamycin, (D) Shoot regeneration of transformed tissue on medium with the addition of kanamycin, (E) Shoot regeneration of non-transformed tissue on medium without kanamycin, (F) Root induction of transformed shoot, (G) Root induction of non-transformed shoot, (H) The acclimatization of transformed (left) and non-transformed (right) plantlets on soil media in plastic pots (bar = 1cm).

3.4. PCR Detection of Transgenic Plant

The results of the PCR analysis of genomic DNA using specific primers of pBI121 forward and *HMGR1* reverse in three transformed plantlets marked TA1, TA2, and TA3 from generation 0 (T0) *A. paniculata* revealed the presence of the *HMGR1* gene, as indicated by the production of a DNA fragment of around 2,262 bp in length (Figure 7A). This corresponded to the length of the *HMGR1* gene in the pBI12-*HMGR1* plasmid (positive control PCR), at 2,262 bp, whereas the negative control contained no *HMGR1* band. The use of pBI121 forward and *HMGR1* reverse was intended to avoid false positives because the *HMGR1* gene was isolated from *A. paniculata* tissue; therefore, if the integration assays of the *HMGR1* gene used the specific *HMGR1* gene, then the non-transformant plants would also produce DNA bands of the *HMGR1* gene. In brief, these results demonstrated the successful transformation of *A. paniculata* with pBI12-*HMGR1* by agroinfiltration. As such, the integration of pBI121-*HMGR1* into the genome of regenerated transgenic plants (T0) was confirmed. However, to prove the stability of a genetic transformation, it is necessary to confirm the presence of inserts in the first, second, and up to third offspring (Chaudhury et al., 2019). Due to resource constraints, we were unable in this present study to verify the stable integration of pBI121-HMGR1 on subsequent offspring, either T1, T2, or T3.

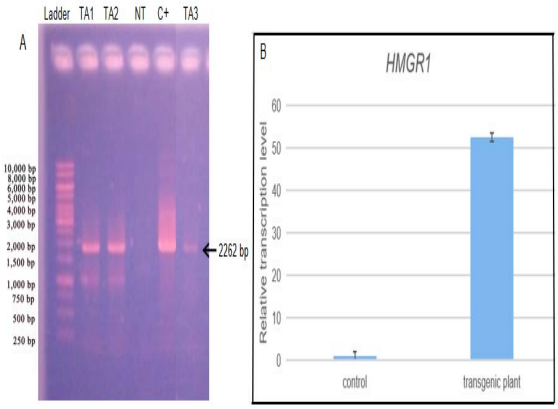

Figure 7. (A) PCR product detection of *HMGR1* gene from transgenic *A. paniculata*.;1Kb: ladder, non-transformant (NT), Positive control (control (+)): specific primers of pBI121 forward and *HMGR1* reverse, transformant repetition 1 (TA1), transformant repetition 2 (TA2), transformant repetition 3 (TA3); (B) Relative transcription level of *HMGR1* gene in control (non-transgenic) and transgenic plant; each transcript level was normalized with respect to the transcript level of the actin gene. Error bars represent the ± SD (standard deviation) of three biological replicates.

3.5. Expression of *HMGR1* Gene

The results showed that the relative transcript level of the *HMGR1* gene in the transgenic plant was significantly higher compared to the non-transgenic plant, specifically 52.5-fold higher (Figure 7B). This indicated that the expression of the *HMGR1* gene under the CaMV 35S promoter was up-regulated. Up-regulation of the *HMGR1* gene was expected to enhance the activity of the HMGR enzyme.

3.6. Up-regulation of HMGR1 Gene Increased the Andrographolide Content

Separate ANOVA tests in this study revealed significantly higher andrographolide levels in transgenic leaf tissue compared to normal leaf tissue, specifically 34.1 mg/g and 11.0 mg/g, respectively. Similarly, the average andrographolide levels in the transformed stems were significantly greater ($P<0.05$) than in normal stems, measuring 6.2 mg/g compared to 2.1 mg/g. Consequently, the transformed plants had a markedly elevated total andrographolide content ($P<0.05$) compared to the normal plants, namely 45.3 mg/g versus 17.6 mg/g. Notably, the andrographolide level in the transformed roots (5.0 mg/g) showed no significant difference from that in the normal roots (4.5 mg/g).

However, a post-hoc Tukey test applied across all tissues indicated no significant differences in the andrographolide levels between the transformed stem tissues, transformed roots, normal leaves, normal stems, and normal roots. Only the transformed leaves showed a noteworthy difference when compared to the transformed stems, transformed roots, normal leaves, normal stems, and normal roots (Table 2).

Table 2. Concentration of andrographolide in leaf, stem, root, and whole plant of the control and transgenic *A. paniculata*

Sample	Average andrographolide concentration* ± SD (mg/g)		Increment (fold)
	control plant	transgenic plant	
leaf	11.0^{a} ±4.1	34.1^{b} ±5.5	3.1
stem	2.1^{a}±0.3	6.2^{a}±2.5	2.9
root	4.5^{a}±0.3	5.0^{a}±3.3	1.1
Total (whole plant)	17.6^{x} ±1.6	45.3^{y}±3.8	2.6

*The concentration of andrographolide in leaf, stem, and root was obtained from control plants and transgenic plants. The data represent an average of three biological replications ± SD (standard deviation). The different superscript letters against the andrographolide concentration numbers for the control and transgenic tissues indicate a significant difference ($P < 0.05$).

4. Discussion

4.1. Construction of pBI121-HMGR1 Plasmid Vector

The pBI121 backbone and *HMGR1* insert were successfully ligated using T4 DNA ligase, producing a recombinant plasmid named pBI121-*HMGR1* with a length of 15,004 bp. By using *HMGR1* forward and reverse primers, the recombinant plasmid was sequenced and analyzed using the BLAST program at NCBI followed by alignment using BioEdit Sequence Alignment Editor version 7.2.5. This demonstrated a high homology between the pBI121-*HMGR1* sequence and *HMGR1 A. paniculata* from the NCBI database, which indicated 99% identity and an expectation (E) value of 0.0. The E value was equal to zero in the BLAST results, indicating a high level of confidence in the analysis results and the presence of a homological relationship between the query sequences and NCBI database sequences (Xiong, 2006). Another study showed that the HMGR gene was successfully constructed into the pBS plasmid, resulting in a pBS-HMGR recombinant that was used as a plant expression vector in *Ligularia fischeri* (Du, *et al.*, 2020).

4.2. Transformation and Plant Regeneration

The transformed cotyledonary tissues of *A. paniculata* survived on the selection medium, i.e. medium containing the antibiotic kanamycin (20 mg/l), whereas non-transformed tissues on the same medium did not develop and eventually died. These results indicate that the surviving tissues were successfully transformed by DNA containing the kanamycin resistance gene that was present in the pBI121-*HMGR1* construct, while the non-transformed tissue was sensitive to kanamycin as these normal tissues did not possess the resistance gene. The integration of the kanamycin resistance gene into the *A. paniculata* genome meant that other genes located in the T-DNA region of the pBI121-*HMGR1* construct, including the *HMGR1* gene and the CaMV35S promoter, also integrated into the *A. paniculata* genome. These results confirmed that the *HMGR1* gene had been inserted into *A. paniculata* tissue. This result is similar to that previously reported in the transformation of *Trachyspermum ammi* mediated by *A. tumefaciens*. The researchers in that study used kanamycin as the antibiotic for tissue selection and

PCR analysis to test for the gene of interest (Nomani and Tohidfar, 2021).

Multiple shoots from the transformed tissue appeared on days 28–30 after initiation in the shoot regeneration medium (MS medium consisting of 2 mg/l BAP and 1mg/l IAA) with the addition of 20 mg/l kanamycin (Figure 6D), while the non-transformed tissue generated multiple shoots faster in a shoot regeneration medium with no added kanamycin, i.e., within 14–16 days after initiation (Figure 6E). It seemed that the addition of kanamycin in the selection medium may have resulted in stress to the transformed tissues, extending the shoot formation time compared to that of the non-transformed tissue. These findings are similar to those reported by Vidal *et al.* (2010).

The average number of regenerated shoots from transformed tissue in the medium with kanamycin added was significantly lower ($P<0.05$) than the number regenerated from non-transformed tissue in the medium with no added kanamycin, i.e. 1.3 compared to 8.0. These results demonstrate that the addition of kanamycin to the medium reduced the number of shoot regenerations. Similar results were reported in the regeneration of transgenic *Citrus aurantifolia* on a medium containing kanamycin as a selective agent, resulting in a low regeneration rate, i.e., 1.3 shoots per explant (Molphe-Balch and Alejo, 1998). Similarly, the number of regenerated shoots from transformed tissue of *Vitis vinifera* cv. Thompson in a medium with the addition of kanamycin was much lower than the number of regenerated shoots from non-transformed tissue in a medium with no added kanamycin, i.e. 4.7 compared to 28 (Sabbadini, *et al*., 2019). These findings demonstrate that sensitivity to the presence of antibiotic-selective agents in a medium strongly influences the number of shoot regenerations. In this context, antibiotics used as selecting agents can lead to necrosis and tissue browning due to the production of reactive oxygen species, which can impact regeneration capacity (Zhou, *et al.*, 2014).

In this study, the regeneration of transformed plant tissues was enabled by direct organogenesis through direct shoot formation from the cotyledon explants followed by root formation to produce transgenic plantlets. Root formation in the transformed and non-transformed shoots (Figure 6F and 6G) was successfully achieved on MS medium with the addition of IBA (1.0 mg/l). These results were in line with Tangapo *et al.* (2012) and Barpete *et al.* (2014) for the promotion of adventitious roots in transgenic shoots whereby an auxin such as IBA was required. Similarly, rooting in *Artemisia* sp. microshoots was successfully achieved in MS medium with the addition of 0.5 mg/l IBA (Shibli, *et al*., 2018). The application of IBA growth hormone effectively induced root formation, since IBA has been widely used in various plants to promote root formation.

4.3. Expression of HMGR1 Gene

The relative expression of the *HMGR1* gene under the CaMV 35S promoter in transgenic *A. paniculata* was 52.5-fold higher compared to in the non-transgenic plant. This is similar to previously reported results that specific promoters can induce structural gene expression (Fu *et al*., 2018). In addition, the promoter CaMV 35S is a strong constitutive promoter used in transgenic expression in plants (Somssich, 2019).

Up-regulating the *HMGR1* gene was expected to enhance the activity of the HMGR enzyme. As reported, the HMGR activity showed a positive correlation with andrographolide biosynthesis in *A. paniculata* (Jha *et al.*, 2011); as such, the increase in HMGR enzyme activity would enhance andrographolide content. Moreover, Jha *et al.* (2011) reported that the application of GA3 and Jasmonic acid enhanced andrographolide by 0.31 fold and 0.56 fold, respectively.

4.4. Up-regulation of HMGR1 Gene Increased the Andrographolide Content

Using a separate ANOVA test, significant differences were observed between the transformed leaf and normal leaf tissues, the transformed stem and normal stem tissues, and the transformed plant and normal plant tissues. Meanwhile, the ANOVA test revealed no significant difference between the transformed and the normal root tissue. However, when advanced tests were conducted to examine differences between groups of samples simultaneously, namely through Tukey's post-hoc test, different results emerged. These revealed no significant differences between the levels of andrographolide in the transformed stem tissues, transformed roots, normal leaves, normal stems, and normal roots. Only the transformed leaves exhibited a notable difference when compared to the transformed stems, transformed roots, normal leaves, normal stems, and normal roots.

It was obvious that the results of this ANOVA test revealed only the presence of differences in the levels of andrographolide in the samples in the normal leaf, normal stem, normal root, transformed leaf, transformed stem, and transformed root tissue, while Tukey's post-hoc test identified the actual groups that differed significantly. This study revealed that the levels of andrographolide in transgenic leaf tissue differed significantly from those in normal leaves, normal stems, normal roots, transformed stems, and transformed roots.

Furthermore, the study demonstrated that andrographolide levels in the transgenic leaves, stems, and roots increased by 3.1, 2.9, and 1.1 times, respectively. Consequently, the average total andrographolide content in the entire transgenic plant was 2.6 times higher than in the normal plant (Table 2). The increased andrographolide levels were primarily driven by a significant increase in the level within the leaf tissue as opposed to in the stem and root tissues. This finding aligns with Royani *et al.* (2014), whose study reported higher andrographolide content in the leaves of *A. paniculata* compared to the stem or root.

The increase in andrographolide levels in the transgenic plant was most likely related to overexpression of the *HMGR1* gene, which has been reported to be 52.5 times higher than in the controls. This aligned with a previous finding that andrographolide biosynthesis was enhanced by 0.31- to 0.6-fold as the expression level of HMGR genes increased (Jha *et al*., 2011). Another study reported that the production of β-sesquiphellandrene increased by 1.25–1.60 fold when the endogenous *HMGR* gene in the eukaryotic mevalonate pathway was overexpressed (Song *et al*., 2012).

Nevertheless, in this study, it appears that the increase in andrographolide concentration was not as high as the increase in *HMGR1* gene expression. This probably reflected how the working position of *HMGR* was at an early stage in the andrographolide biosynthesis pathway via the mevalonate pathway, while andrographolide synthesis occurs further downstream of the pathway, with several steps still required before reaching the andrographolide synthesis stage (Figure 8). In andrographolide biosynthesis via the mevalonate cascade, several intermediate compounds are involved, including mevalonate, FPP (farnesyl diphosphate), and GGPP (Geranyl-geranyl diphosphate), as described by Jha *et al*. (2011). These intermediate compounds are used as the precursors of many compounds; for example, FPP is used as a precursor for the synthesis of sesquiterpenes and triterpenes, GGPP is used not only for the synthesis of andrographolide (diterpene lactone) but also for the biosynthesis of other diterpenes such as ent-kaurene and Gibberellins and for the synthesis of polyterpenes (Taiz and Zeiger, 2002). Consequently, the substrate for the synthesis of andrographolide was reduced. This potentially explains why the increase in andrographolide content was not as high as the increase in *HMGR1* gene expression in *A. paniculata*.

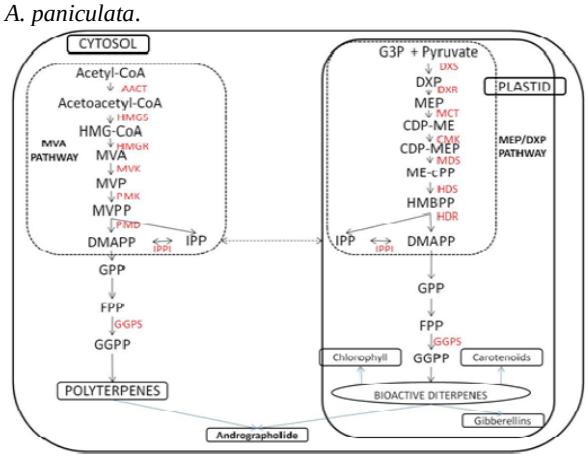

Figure 8. Biosynthetic pathway of andrographolide (Jha *et al*., 2011)

5. Conclusions

Andrographis paniculata tissue was successfully transformed by recombinant plasmid pBI121-*HMGR1* using the *Agrobacterium tumefaciens* GV3101-mediated transformation procedure. The overexpression of *HMGR1* under the CaMV 35S promoter in the whole plant enhanced the andrographolide content by up to 2.6 times compared to the control plant. This demonstrates that the amount of andrographolide was associated with the expression level of the *HMGR1* gene in the andrographolide biosynthetic pathway. This study is the first to report that overexpression of the *HMGR1* gene under the CaMV 35S promoter in the *A. paniculata* medicinal plant can enhance the principal active constituent andrographolide. This finding is, therefore, beneficial for biotechnological research in medicinal plants.

Acknowledgement

This research was funded by the Research, Community Services, and Innovation Program of Institut Teknologi Bandung, grant number 2019/I1.C02.2/KU/2019.

References

Akbar S. 2011. *Andrographis paniculata:* A review of pharmacological activities and clinical effects. Altern Med Rev., **6(1)**:66–77. https://www.researchgate.net/publication/50865161_Andrographis_paniculata_A_Review_of_Pharmacological_Activities_and_Clinical_Effects

Allison LA. 2007. **Fundamental Molecular Biology** 1st edition. Blackwell Publishing, Oxford. https://molbiomadeeasy.files.wordpress.com/2013/09/fundamental_molecular_biology.pdf

Alvarez MA, Marconi PL. 2011. Genetic transformation for metabolic engineering of tropane Alkaloids in **Genetic Transformation** E-book. https://doi.org/10.5772/34216

Amack SC, Antunes MS. 2020. CaMV35S promoter–A plant biology and biotechnology workhorse in the era of synthetic biology. Curr. Plant Biol., **24**: 100179. https://doi.org/10.1016/j.cpb.2020.100179

Amara AA. 2018. Fast plasmid slot lysis and gram-negative bacteria ghost preparation protocol. Austin J. proteom bioinfo & genom., **5(1)**:1025. https://www.researchgate.net/publication/328841226

Barpete S, Khawar KM, Özcan S. 2014. Differential competence for in vitro adventitious rooting of grass pea (*Lathyrus sativus* L.). PCTOC., **119(1)**:39–50. https://doi.org/10.1007/s11240-014-0512-6

Benoy GK, Animesh DK, Aninda M, Priyanka DK, Sandip H. 2012. An overview on *Andrographis paniculata* (Burm.F.) Nees. Int J Res in Ayurveda and Pharm., **3(6):**752–760. https://doi.org/10.7897/2277-4343.03610

Calabrese C, Berman SH, Babish JG. 2000. A phase I trial of andrographolide in HIV positive patients and normal volunteers. Phytotherapy Res., **14(5)**:333–338. https://doi.org/10.1002/1099-1573(200008)14:5<333::aid-ptr584>3.0.co;2-d.

Chandrasekaran C, Murali B, Deepak M, Agarwal A. 2012. In vitro comparative evaluation of non-leaves and leaves extracts of *Andrographis paniculata* on modulation of inflammatory mediators. Anti-Inflam & Anti-Allerg Agents in med Chem., **11(2):**191-197. https://doi.org/10.2174/187152312803305731.

Chang AY, Chau VWY, Landas JA, Pang Y. 2017. Preparation of calcium competent *Escherichia coli* and heat-shock transformation. JEMI-methods., **1**: 22-25. https://ujemi.microbiology.ubc.ca/sites/default/files/Chang%20et%20al%20JEMI-methods%20Vol%201%20pg%2022-25.pdf

Chao WW, Lin BF. 2010. Isolation and identification of bioactive compounds in *Andrographis paniculata* (Chuanxinlian). Chin Med., **5(17)**: 1-15. https://doi.org/10.1186/1749-8546-5-17

Chaudhury A, Dalal AD, Sheoran NT. 2019. Isolation, cloning and expression of CCA1 gene in transgenic progeny plants of Japonica rice exhibiting altered morphological traits. PLOS ONE., **14(8)**. https://doi.org/10.1371/journal.pone.0220140.

Churiyahi, Pongtulurani OB, Rofaani E, Tawardi. 2015. Antiviral and Immunostimulant Activities of *Andrographis paniculata.* HAYATI J Biosci., **22(2)**: 67-72. https://doi.org/10.4308/hjb.22.2.67

Dong N, Ponciano G, McMahan CM, Coffelt TA, Johnson L, Creelman R, Whalen MC, Cornish K. 2013. Overexpression of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Parthenium argentatum.* Ind Crops Prod., **46**:15-24. https://doi.org/10.1016/j.indcrop.2012.12.044

Du J, Wenzhong Hu W, Yang D, Meng F, , Li Y, 2020. Cloning and functional analysis of HMGR gene in *Ligularia fischeri.* IOP Conf. Ser.: Earth Environ. Sci., 615:012112. https://doi.org/10.1088/1755-1315/615/1/012112

Enmozhi SK, Raja K, Sebastine I, Joseph J. 2020. Andrographolide as a potential inhibitor of SARS-CoV-2 main protease: An in silico approach. J Biomol Struct Dyn., **39(9)**:3092-3098. https://doi.org/10.1080/07391102.2020.1760136.

Fu R, Martin C, Zhang Y. 2018. Next-Generation Plant Metabolic Engineering Inspired by an Ancient Chinese Irrigation System. Mol. Plant **11(1)**: 47–57. https://doi.org/10.1016/j.molp.2017.09.002

Han JY, Kim MJ, Ban YW, Hwang HS, Choi YE. 2013. The involvement of b-Amyrin 28-oxidase (CYP716A52v2) in oleanane-type ginsenoside biosynthesis in Panax ginseng. Plant Cell Physiol., **54(12)**: 2034–2046. https://doi.org/10.1093/pcp/pct141

Han L, Li J, Guo J, Ma Y, Guo X. 2017. Expression vector construction and genetic transformation of *Paeonia lactiflora* gibberellin 20-oxidase gene. Am. J. Plant Sci., **8(7)**: 1525-1533. https://doi.org/10.4236/ajps.2017.87105

Harjotaruno S, Widyawaruyantil A, Zaini NC. 2008. Apoptosis inducing effect of andrographolide on TD-47 human breast cancer cell line. AJTCAM., **4(3)**:345–351. https://doi.org/10.4314/ajtcam.v4i3.31228

Jarukamjorn K, Nemoto N. 2008. Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide. J. Health Sci., **54(4)**:370–381. https://doi.org/10.1248/jhs.54.370

Jha Z, Sharam SH, Sharma DK. 2011. Differential expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase of *Andrographis paniculata* in andrographolide accumulation. J. Chem. Pharm. Res., **3(3)**:499-504. https://www.jocpr.com/articles/differential-expression-of-3hydroxy3methylglutarylcoenzyme-a-reductase-of-andrographis-paniculata-in-andrographolide-acc.pdf

Kabir MH, Hasan N, Rahman MM. 2014. A survey of medicinal plants used by the Deb barma clan of the Tripura tribe of Moulvibazar district, Bangladesh. J. Ethnobiol. Ethnomed., **10(19)**:1-28. https://doi.org/10.1186/1746-4269-10-19.

Kim YJ, Lee OR, Oh JY, Jang MG, Yang DC. 2014. Functional analysis of 3-hydroxy-3-methylglutaryl coenzyme-a reductase encoding genes in triterpene saponin producing ginseng. Plant Physiol., **165(1)**:373–387. https://doi.org/10.1104/pp.113.222596

Koerniati S, Widhianata H. 2012. Construction and transformation of HVA1 gene expression vector into Indoensian elite rice varietis. J. AgroBiogen., **8(2)**:54-6. https://doi.org/10.21082/jbio.v8n2.2012.p54-61

Konagaya KI, Nanasato Y, Taniguchi T. 2020. A protocol for Agrobacterium-mediated transformation of Japanese cedar, Sugi (*Cryptomeria japonica* D. Don) using embryogenic tissue explants. Plant Biotech., **37(2)**:147–156. https://doi.org/10.5511/plantbiotechnology.20.0131a

Kumar RA, Sridevi K, Vijaya KN, Nanduri S, Rajagopal S. 2004. Anticancer and immunostimulatory compounds from *Andrographis paniculata*. J. Ethnopharmacol., **92(2-3)**:291–295. https://doi.org/ 10.1016/j.jep.2004.03.004.

Laksmiani NPL, Larasanty LPF, Santika AAGJ, Prayoga PAA, Dewi AAIK, Dewi NPAK. 2020. Active Compounds Activity from the Medicinal Plants Against SARS-CoV-2 using in Silico Assay. Biomed. Pharmacol. J., **13(2)**:73–881. https://dx.doi.org/10.13005/bpj/1953

Lakshmi SA, Shafreen RMB, Priya A, Shunmugiah KP. 2021. Ethnomedicines of Indian origin for combating COVID-19 infection by hampering the viral replication: using structure-based drug discovery approach. J. Biomol. Struct. Dyn., **39(13)**:4594-4609. https://doi.org/ 10.1080/07391102.2020.1778537.

Levita J, Nawawi A, Mutholib A, Ibrahim S. 2010. Andrographolide: a review of its anti-inflammatory activity via inhibition of NF-kappaβ activation from computational chemistry aspects. Int J. Pharmacol., **6(5)**:569–76. https://doi.org/10.3923/ijp.2010.569.576

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2-ΔΔCT method. Methods., **5(4)**:402–408. https://doi.org/10.1006/meth.2001.1262

Lu X, Tang K, Li P. 2016. Plant metabolic engineering strategies for the production of pharmaceutical terpenoids. Front. Plant Sci., **7**:1647. https://doi.org/10.3389/fpls.2016.01647

Majee C, Gupta BK, Mazumder R, Chakraborthy GS. 2011. HPLC method Development and Characterization of Bio-Active Molecule Isolated from *Andrographis paniculata.* Int. J. Pharmtech Res., **3(3)**: 1586-1592.

Marwani E, Tangapo A, Dwivany FM. 2013. Agrobacterium-mediated stable transformation of medicinal plant *Andrographis paniculata* callus expressing ß-glucuronidase (GUS) gene. IJB., **18 (2)**: 92-100. https://doi.org/10.22146/ijbiotech.7873.

Menra JP. 2013. Isolation and construction of 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 gene (*HMGR1*) from *Andrographis paniculata* (Burm.f.) Wallich. ex Nees . [Unpublished master's thesis]. Institut Teknologi Bandung.

Mohammed S, Samad AA, Rahmat Z. 2019. Isolation, Cloning, and Sequence Analysis of the Full-Length RFT1 Gene from Malaysian Upland Rice (*Oryza sativa*, subsp. Indica, Cultivar Wai). JJBS., **12(3):** 259-265**.** https://jjbs.hu.edu.jo/files/vol12/n3/Paper%20number%202.pdf

Molphe-Balch EP, Ochoa-Alejo N. 1988. Regeneration of transgenic plants of Mexican lime from *Agrobacterium rhizogenes*-transformed tissues. Plant Cell Rep., **17**: 591–596. doi: 10.1007/s002990050448

Nomani M, Tohidfar M. 2021. Plant regeneration and transformation of *Trachyspermum ammi* using *Agrobacterium tumefaciens* and zygotic embryos. JGEB., **19(1)**: 1-10. https://doi.org/10.1186/s43141-021-00173-8

Putter KM, Deenan NV, Unland K, Prufer D, Gronover CS. 2017. Isoprenoid biosynthesis in dandelion latex is enhanced by the overexpression of three key enzymes involved in the mevalonate pathway. BMC Plant Biol., **17(88)**:1-13. https://doi.org/10.1186/s12870-017-1036-0

Rao KY, Vimalamma G, Rao CV, Tzeng YM. 2004. Flavonoids and andrographolides from *Andrographis paniculata.* Phytochem., **65(16)**:2317-2321. https://doi.org/10.1016/j.phytochem.2004.05.008

Royani JI, Hardianto D, Wahyuni S. 2014. Analisis kandungan andrographolide pada tanaman sambiloto (*Andrographis paniculata*) dari 12 lokasi di Pulau Jawa. JBBI., **1(1)**:15-20. https://doi.org/10.29122/jbbi.v1i1.547

Sabbadini S, Capriotti L, Molesini B, Pandolfini T, Navacchi O, Limera C, Ricci A, Mezzetti B. 2019. Comparison of regeneration capacity and *Agrobacterium*-mediated cell

transformation efficiency of different cultivars and rootstocks of Vitis spp. via organogenesis. Sci Rep., **9**: 582.

doi: 10.1038/s41598-018-37335-7

Sharma SN, Jha Z, Sinha RK. 2013. Establishment of in vitro adventitious root cultures and analysis of andrographolide in *Andrographis paniculata*, Nat Prod Cummon., **8(8)**:1045-7. : https://www.researchgate.net/publication/256196934

Shibli RA, Sharaf SA, Kasrawi MA, Al-Qudah T. 2018. In Vitro Multiplication of the White Wormwood, Artemisia. JJBS., **11(3)**: 265-271.
https://jjbs.hu.edu.jo/files/v11n3/Paper%20Number%205.pdf

Shi TH, Huang YL, Chen CC, Pi WC, Hsu YL, Lo LC, Chen WY, Fu SL, Lin CH. 2020. Andrographolide and its fluorescent derivative inhibit the main proteases 2019-nCoV and SARS-CoV through covalent linkage. BBRC., **533(3)**:467-473. https://doi.org/10.1016/j.bbrc.2020.08.086

Shim JS, Lee OR, Kim YJ, Lee JH, Kim JH, Jung DY. 2010. Overexpression of PgSQS1 increases ginsenoside production and negatively affects ginseng growth rate in Panax ginseng. JGR., **34(2)**:98-103. https://doi.org/10.5142/jgr.2010.34.2.098

Somssich, M. 2019. A short history of the CaMV 35S promoter. PeerJ Prepr., **7**: e27096v3. https://doi.org/10.7287/peerj.preprints.27096v3

Song AAL, Abdullah JO, Abdullah MP, Shafee N, Othman R, Tan EF, Noor NM, Raha AR. 2012. Overexpressing 3-Hydroxy-3-Methylglutaryl coenzyme A reductase (HMGR) in the Lactococcal mevalonate pathway for heterologous plant sesquiterpene production. PLos One., **7**: e52444. https://doi.org/10.1371/journal.pone.0052444

Suebsana S, Pongnaratorn P, Sattayasai J, Arkaravichien T, Tiamkao S, Aromdee C.2009. Analgesic, antipyretic, anti-inflammatory, and toxic effect of andrographolide derivates in experimental animal. Arch Pharm Res., **32(9)**:1191-1200. https://doi.org/10.1007/s12272-009-1902-x.

Sukumaran S. 2011. Concentration determination of nucleic acids and proteins using the micro-volume bio-spec nano spectrophotometer. JoVE., **48**: 1-5. https://doi.org/10.3791/2699.

Tangapo AM, Marwani E, Dwivany FM. 2012. Transformasi dan ekspresi transien gen pelapor gus pada *Andrographis paniculata* (Burm.F.) Wallich Ex Ness or transformation and expression of reporter gene Gus on *Andrographis paniculata* (Burm.F.) Wallich Ex Ness. JBL., **2(1)**: 1-10. https://doi.org/10.35799/jbl.2.1.2012.375

Taiz L and Zeiger E. 2002. **Plant Physiology**, 5th Edition. Sinauer Associates Inc., Sunderland. https://www.academia.edu/27225799/Plant_Physiology_Taiz_and_Zeiger_

Vidal N, Mallon R, Valladares S, Meijomin AM. 2010. Regeneration of transgenic plants by Agrobacterium-mediated transformation of somatic embryos of juvenile and mature *Quercus robur* Abbreviations AS Acetosyringone BA Benzyladenine Kan Kanamycin MS Murashige and Skoog NAA a-Naphthaleneacetic acid X-Gluc 5-Bromo-4-chloro-3-indolyl-b-D-glucuronide. Plant Cell Rep., **29(12)**: 1411-1422. https://doi.org/10.1007/s00299-010-0931-8

Wiart C, Kumar K, Yusop MY, Hamimah H, Fauzi ZM, Sulaiman M. 2005. Antiviral properties of Ent-labdene diterpenes of *Andrographis paniculata* Nees, inhibitors of herpes simplex virus type 1. Phytother Res., **19(12)**:1069-1070. https://doi.org/10.1002/ptr.1765

Xiong J. 2006. **Essential Bioinformatics**. Cambridge: Cambridge University Press. https://books.google.co.id/books?id=92K1QgAACAAJ&hl=id

Zhou Q, Dai L, Cheng S, He J. 2014. A circulatory system useful both for long-term somatic embryogenesis and genetic transformation in *Vitis vinifera* L. cv. Thompson Seedless. PCTOC., **118(1)**:157–168. doi: 10.1007/s11240-014-0471-y.