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

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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 into 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), anticaner (Harjotaruno et al., 2008), antidiabetic, antimarial, 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 (Wiert et al., 2005), as well as antipyretic, immunostimulant, analgesic, anticancer (Suesbana 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; Emmozhi et al., 2020; Laksmani 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
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 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 (Jha et al., 2016). 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 brevicornuculatum 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 pBH121 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 pBH121 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 CGS 3’ and reverse primer 5’ATC GAC TCT CTC TGT CTC CAA TCT CAA GTG 3’, 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°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 XbaI, BamHI, SmaI, XhoI, and BsrGI restriction sites between the CaMV 35S promoter and GUS gene. Of the five restriction enzyme recognition sites, XbaI and BsrGI do not cleave the HMGR1 gene. Therefore, a pair of primers was designed with XbaI (5’-TCT AGA AGA ATG GCC GCC CTC CGC CGC CGA) and BsrGI (TGT ACA AAT CGA CTC TCT CTG TC T 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 XbaI and BsrGI, 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 XbaI 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).

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 XbaI and BsrGI 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 ATG GTG GAG CA and Skoog (MS) medium to reach a concentration of 10^6 cells/ml or OD600nm = 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 regenerating 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 Tiangen. 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 TCT AGA AGA ATG GCC GCC CTC CGC CGC CGA-3′, giving an 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, elongation at 72°C for 2 minutes, and final elongation at 72°C for 7 minutes. The PCR product was 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.

2.9. 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 A260/A280 ≥ 1.8 was selected due to its high purity, as stated by Sukumaran et al. (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 HMG1 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-ΔΔCt 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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Product size</th>
</tr>
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<tbody>
<tr>
<td>Actin-F</td>
<td>5’GAGGTGCTCTTTCAGCCATC’3</td>
<td>250bp</td>
</tr>
<tr>
<td>Actin-R</td>
<td>5’TGTACCTTCTAGTGGCTGGG’3</td>
<td></td>
</tr>
<tr>
<td>HMGFR-F</td>
<td>5’GCTACTGTTTGTCCAGTCT’3</td>
<td>250bp</td>
</tr>
<tr>
<td>HMGFR-R</td>
<td>5’GAAGAGCCGATTTCGCAGTC’3</td>
<td></td>
</tr>
</tbody>
</table>

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 of A. paniculata were harvested from the age of 3 months. The cDNA fragment (around 1,800 bp) isolated from A. paniculata was verified as the size of HMG1 gene (Figure 2C).

The cDNA fragment (around 1,800 bp) isolated from A. paniculata was verified as HMG1 by DNA sequencing (GenBank accession nos. AY429658.1). The insertion of the Hma and BsrGI restriction enzyme site to the HMG1 fragment produced a fragment measuring between 1,500 bp and 2,000 bp in length (Figure 2A), which corresponded to the expected Hma-HMG1- BsrG1 gene (1,822 bp). The digestion of pBl121 with Hma and BsrG1 restriction enzymes at specific nucleotide sequences eliminated the GUS gene and produced the pBl121 backbone with a length of 13,182 bp (Figure 2B), whereas digesting the pGEM-T Easy-HMG1 with the same restriction enzymes produced a DNA fragment between 1,500 bp and 2,000 bp in length, which corresponded to the HMG1 gene (Figure 2C).

Figure 2. (A) HMG1 gene fragment containing Hma and BsrG1 restriction sites with a length of 1,500 bp–2,000 bp, corresponding to the size of HMG1 (1,822 bp). (B) Gel electrophoresis of pBl121 digested with Hma and BsrG1 restriction enzymes, which produced the pBl121 backbone with a length of 13,182 bp, indicated by the red box. (C) Gel electrophoresis of pGEM-T Easy-HMG1 digested with Hma and BsrG1 restriction enzymes, which produced a fragment with a length of 1,500 bp–2,000 bp, corresponding to the size of HMG1 (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.](image)

**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).

![Figure 4.](image)

**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 pBI121-*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.](image)

**Figure 5.** (A) Colonies of *A. tumefaciens* harboring recombinant plasmid pBI121-*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.
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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average andrographolide concentration* ± SD (mg/g)</th>
<th>Increment (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control plant</td>
<td>transgenic plant</td>
</tr>
<tr>
<td>leaf</td>
<td>11.0±4.1</td>
<td>34.1±5.5</td>
</tr>
<tr>
<td>stem</td>
<td>2.1±0.3</td>
<td>6.2±2.5</td>
</tr>
<tr>
<td>root</td>
<td>4.5±0.3</td>
<td>5.0±3.3</td>
</tr>
<tr>
<td>Total (whole plant)</td>
<td>17.6 ±1.6</td>
<td>45.3 ±3.8</td>
</tr>
</tbody>
</table>

*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 homologous 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 (Noman 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 aurantiifolia 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 Viitis 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 applications 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 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 (Taitz 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)](https://example.com/figure8.png)

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.

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References


