Identification, Expression, Phylogenetic Analysis of 3-hydroxy-3-methylglutaryl-CoA reductase in *Persicaria odorata* Under Wounding Treatment

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Abstract

HMGR is the first rate-limiting step is mevalonate pathway for terpenoid biosynthesis. Expression of HMGR gene in plant is important as the high or low expression will influence the production of terpenoid, valuable secondary metabolites. Previous study has shown the expression of HMGR gene can be influenced by environmental factors such as biotic and abiotic stress. However, the effect of biotic stress especially wounding towards HMGR gene expression in *Persicaria odorata* remain unclear. Moreover, the evolutionary relationship of the HMGR gene in *P. odorata* also still unknown. Investigating this relationship may provide insight about the function of the gene. Therefore, this study is carried out to identify the response of HMGR gene against wounding stress and its evolutionary relationship across plant species. In this study, two *Persicaria odorata* plants were prepared, leaves of one plant was wounded using needle and another plant is kept as control. The leaves from both plants were sampled as control sample and treated or wounded samples. RNA was extracted from both plants and cDNA was synthesized. Gene expression analysis was carried out using RT-PCR and analysing gel electrophoresis image using Image J. Furthermore, the phylogenetic analysis of HMGR was done using Mega software. Based on the findings, the concentration of extracted RNA for control plant and wounded plant was 654.6 ng/ µL and 758.45ng/ µL respectively at ultraviolet absorbance 260 nm. RNA integrity check showed intact RNA for both samples due to the presence of 28S and 18S rRNA. From the gene expression analysis, untreated *Persicaria odorata* has an expression value of 2.96 and it increased to 5.88 for wounded *Persicaria odorata.* The maximum likelihood tree was constructed using *Kimura-2-parameter* model. The phylogenetic tree revealed that HMGR from *Persicaria* obtained from NCBI database has the closest relationship and HMGR in *Ipomoea nil* with 3 nodes apart, but the low bootstrap value, 19 indicates insignificant relationship between them. Overall, these findings revealed that HMGR gene in *Persicaria odorata* is upregulated in response to biotic stress and has closest relationship with HMGR from *Ipomoea nil.*

Keywords: *Persicaria odorata*; wounding; HMGR; gene expression; evolutionary relationship

Introduction

Persicaria odorata is a type of herb found in Southeast Asia countries and commonly used for cooking. This herb is found to have many beneficial medicinal effects such as antimicrobial, antiviral, antioxidant, anti-inflammatory, anti-cancer (Halim et al., 2021). Due to its pharmaceutical activities, *P. odorata* has been widely researched for its chemical and constituents' properties. Various parts of *P. odorata* has been studied such as leaves, stems and aerial parts and extracted through water, methanol and ethanol as essential oil or extracts (Halim et al., 2021; Nguyen, 2020).

Plants produce a vast diversity of secondary metabolites in nature including *P. odorata.* Various research has proved that the presence of secondary metabolites in plants are the compounds responsible for its medicinal effects. Terpenoids is a class of secondary metabolites contributed to the

medicinal properties of *P. odorata* (Hussein & El-Anssary, 2019). Secondary metabolites are biosynthesized in association with primary metabolism as part of plants' developmental physiology and defence mechanism. The secondary metabolites protect the plants when facing pest, disease, and environmental stress (Hussein & El-Anssary, 2019). In nature, it will be synthesized in low quantities and stored in parts of plants.

However, the biosynthesis of secondary metabolites in plants are found to be up-regulated in response to various stress due to abiotic or biotic factors (Tan et al., 2019). Abiotic factors are stressors from environment such as drought, temperature, pH and salinity, whereas biotic factors are pathogen attacks from living organism such as bacteria, fungi, insects. Physical wounding is one of biotic factors as it will mimic the pathogenic attack in plants. These stresses trigger plant defence mechanism which activates terpenoid biosynthesis through two pathways; mevalonate pathway in cytosol and non-mevalonate pathway in plastid. In mevalonate pathway, one of the rate-determining steps for biosynthesis of secondary metabolite is HMG-CoA reductase (HMGR) which catalyzes the reaction from HMG-CoA to mevalonate.

With the development in genomics, many research has been done to study the effect of different types of stressors for the regulation of HMGR gene expression. The HMGR gene expression regulates the synthesis of terpenoid in plants (Qing et al., 2020; Sanchez et al., 2017; Hanim et al., 2016). HMGR is an enzyme responsible for catalysing mevalonate from HMG-coa in mevalonate pathway. Investigation on HMGR gene is important as it is the rate-limiting enzyme that produces the main compounds in *Persicaria odorata,* terpenoid. However, the presence of HMGR in kesum species, *P. odorata* is poorly understood.

In addition, previous study showed the level of HMGR are affected by environmental stress. There are two types of stresses able to trigger terpene biosynthesis; abiotic factors and biotic factors. Wounding in plants can occurreddue to pathogen attack, mechanical stress, damage from wind and others (Sabina & Jithesh, 2020). These wounding break down cellular integrity and membrane, trigger plant defence mechanism such as terpenoid biosynthesis (Myers et al., 2022). Previous study showed that a biotic elicitor, *E. coli* had upregulates the HMGR expression in *Helicteres isora* which enhances its secondary metabolites content (Shaikh et al., 2020). Moreover, the evolutionary relationship of the HMGR gene in *P. odorata* also still unknown. This phylogenetic analysis is important to find the conservation of the gene, in which the data can be used to predict the gene function.

Therefore, this research is carried out to detect the presence of HMGR gene in *P. odorata*. Besides, the level of HMGR gene expression against wounding stresses will be investigated to gain insight of function of HMGR under stresses. In addition, the phylogenetic analysis will be conducted to investigate the evolutionary relationship of HMGR gene in *P. odorata* with HMGR genes from other plant species.

Materials and methods

Two *P. odorata* plants were planted for 4-6 weeks in black soil at AgroTani UTM. Then, one of the plants was physically wounded by making punctures on each leaves using needle, while another healthy plant was not treated and kept as control. Both plants were left for 48 hours (Samad, 2019). After 48 hours, sampling of plant materials was done by collecting leaves of control and treated plant into falcon tubes and kept in liquid nitrogen. The plant materials were stored in -80°C freezer until needed. To reduce the risk of RNA degradation by RNases, diethylpyrocarbonate (DEPC) treated water was prepared to treat the apparatus and as solvent to make solution for RNA extraction (Zhu et al., 2020). 1 mL of 0.1% cold DEPC was added to 1000 mL of distilled water in a conical flask. The mixture was stirred using magnetic stirrer for 12 hours followed by autoclaving at 121° C for at least 30 minutes. All the glassware, spatula. mortar and pestle were soaked overnight with DEPC-treated water and discarded, followed by autoclaving at 121° C for at least 30 minutes.

To make 5 M sodium chloride, 29.2 g of NaCl was added into the treated Scott bottle and unautoclaved DEPC water was added until 100 mL. The mixture was stirred using magnetic stirrer and left overnight. The mixture was autoclaved on the next day. The treated mortar and pestle were wrapped

with aluminum foil and autoclaved. Then, the mortar and pestle were baked in oven for at least 4 hours at 150° C.

RNA extraction was conducted using PureLink® Plant RNA Reagent according to manufacturer's protocol. The mortar and pestle were cooled using liquid nitrogen to minimize risk of RNA degradation. Chemicals used have been placed in ice box; 4 °C, while the samples placed in liquid nitrogen   Sample was ground using mortar and pestle in liquid nitrogen until it became very fine powder. Around 0.1 g of leaf was added into 1.5 mL microcentrifuge tube followed by 0.5 mL of cold Pure-Link® Plant RNA Reagent. The sample was vortexed using vortex mixer until sample was homogenized. Then, the tube was laid horizontally and incubated for 5 minutes at room temperature and subsequently centrifuged at 12,000 g for 2 minutes at 4 °C. Then, the supernatant was transferred into a new microcentrifuge tube, followed by the addition of 0.1 mL of 5M NaCl and 0.3 mL of chloroform. The sample was mixed by inverting and tapping the tube. Next, the sample was centrifuged at 12,000 x g for 10 minutes at 4 °C. The supernatant was transferred into a new microcentrifuge tube and mixed with equal volume of isopropanol. The sample was left for 10 minutes at room temperature, followed by centrifuged at 12,000 xg for 10 minutes at 4°C. The resulting supernatant was discarded using micropipette and 1 mL of 75% ethanol was added into the tube containing pellets. After that, the sample was centrifuged at 12,000 xg for 1 minute at room temperature, then supernatant was discarded which is ethanol using micropipette. The sample pellets were briefly centrifuged, and residual ethanol was removed again using micropipette. The microcentrifuge tube was left open for 1 minute to allow ethanol to evaporate and dry the pellets. Lastly, the sample was diluted with 20 μL of RNase- free water and resuspend. The extracted RNA was stored in -80 °C. The treated plant sample was done using the same procedure as above.

The RNA quantity was determined based on concentration and purity of obtained RNA samples measured using Nanodrop spectrophotometer (ND-1000). The Nanodrop sensor was cleaned using Kimwipe before and after each usage. One drop of RNase-free water was carefully pipetted on the sensor and set as blank. Next, the sample was loaded on the sensor and measured. The absorbance of samples was measured at wavelength 230 nm, 260 nm and 280 nm. Then, the 260/280 and 260/230 ratio were determined using Thermo Scientific NanoDrop™ 1000 spectrophotometer software to get the RNA purity.

The RNA quality was determined by examine the bands on gel for intact RNA or denatured RNA on 1% agarose gel (Thermo Fisher Scientific - US, n.d.), 1% agarose gel was made with dissolved agarose gel powder with 1X TAE buffer using a microwave until a clear solution was obtained. The liquid agarose gel was cooled before addition of 4 μL of staining solution; GreenSafe DNA Gel Stain. The mixture was swirled gently and poured into electrophoresis tray and comb was placed. The gel was left to solidify for around 30 minutes. Next, the comb was removed, the agarose gel was placed in the casting tray and TBE buffer poured to cover the agarose gel. 1 μg of RNA samples was mixed with 6X nucleic acid loading dye on a parafilm to get final concentration of 1X. The mixture is loaded into gel wells. Gel electrophoresis was done for 60 minutes at 90 V. The agarose gel is visualized using Bio-Rad Imaging system.

First strand cDNA synthesis was performed by reverse transcription using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. The RNA samples and reagents used in cDNA synthesis were thawed in ice. After samples were thawed, briefly centrifuge samples and reagents. The process of adding reagents was done on ice. 3 ng of RNA from each sample was pipetted into PCR tubes. 1μL of Oligo (dT)$_{18}$ primer was added, followed by nuclease free water until the volume of mixture reached 12 μL. Next, 4 μL of 5X Reaction Buffer, 1 μL RiboLock RNase Inhibitor (20 U/μL), 2 μL of 10 mM dNTP Mix, and 1 μL of RevertAid M-MuLV RT (200 U/μL) were subsequently added into PCR tubes. The samples were mixed gently and centrifuged briefly. Samples were incubated for 60 minutes at 42 °C using heat block for cDNA synthesis. Then, the samples were incubated at 70°C for minutes to deactivate the enzyme to terminate polymerization reaction. The cDNA samples were stored at 80°C.

The forward and reverse primers for the target gene and reference gene was designed using Primer3Plus tool (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and manufactured by Integrated DNA Technologies (M. Park, Won, Choi, & Lee, 2020). The nucleotide sequence HMGR

and tubulin gene were searched from NCBI database (https://www.ncbi.nlm.nih.gov/). Nucleotide sequences were copied and pasted in a document, region consisted 100 bases flanked binding domain was copied and inserted into Primer3Plus tools in FASTA format. The optimal parameters were set to get good primers. Information of primers designed was summarized in Table 1.

Table 1: Information of primers designed.

Target gene and reference gene	Primer sequences		Size of amplicon (bp)	Melting temperature
	Forward primer	Reverse primer		
HMGR	CTTGGTGGGTTC AATGCTCA	ACCAACTGTTCCGACCT CAA	180	57 °C/ 55.5 °C
Tubulin	TACCAGCCACCA ACCGTAGTCC	CCAACCTCCTCGTAGTC TTTCTCAA	200-300	60.9°C/ 58°C

To verify the effectiveness of designed primer in amplifying targets, RT-PCR was performed using NEB One*Taq*® 2X Master Mix with Standard Buffer following the recommended protocol. The cDNA samples, primers and reagents were kept in ice. The samples, primers and reagents were briefly centrifuged. The procedure of adding reaction components was conducted on ice. 12.5 µL of One*Taq*® Quick Load 2X Master Mix with Standard Buffer was added into a PCR tube. Then, 0.5 5 µL, of forward and reverse primers for HMGR was added, followed by cDNA of control sample. Nuclease free water was added until the reaction volume reached 25 µL. The mixture was briefly centrifuged. Polymerase Chain reaction was done using thermocycler with the conditions as shown in Table 3.2.
The PCR products were analyzed with gel electrophoresis as mentioned in 3.2.4. 3% agarose gel was used for the gel electrophoresis. The same procedure was done using treated sample and also forward and reverse primers for reference gene. The annealing temperature of reference gene was 55°C while other thermocycling conditions remained.

The sequence of HMGR gene was obtained from NCBI. Then, the sequence was BLAST in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). 30 sequences were selected with the highest similarity in query cover and downloaded. Multiple sequence alignment was done using Clustal W in Molecular Evolutionary Genetics Analysis (MEGA) software version 11(Tamura et al., 2021). Next, the sequence that did not overlap with query sequence were trimmed and the alignment was exported in MEGA format.   To find the best substitution model, analysis preference was selected. The lowest value for Bayesian information criterion (BIC) or Akaike information criterion (AICc) and its corresponding model were determined which is *Kimura-2-parameter* model. The phylogeny tree was constructed using maximum-likelihood tree. The test of phylogeny was done using the bootstrap method with 1000 bootstrap replications. The rate among sites was gamma distributed. Then, the maximum likelihood phylogeny tree was constructed.

Results and discussion
The extracted RNA from both plant sample control and treated was analyzed for its concentration and purity. Table 2 show the concentration of control sample is 654.6 ng /µL while treated sample has concentration of 758.45 ng/ µL. The value of control and treated samples for absorbance ratio; $A_{260/280}$ and $A_{260/230}$ is almost the same. $A_{260/280}$ of control sample is 2.105 while treated sample is 2.09. Both readings are approximately 2 which indicates samples are less contaminated by protein which may come from protein of plants (Ahlberg et al., 2021).

$A_{260/230}$ is a secondary measurement for RNA purity. The ratio for control and treated samples were 1.75 and 1.655 respectively. These readings are lower than the pure nucleic acid range 2.0-2.2, thus it indicates the presence of salt or organic compounds in the samples. This is because chemical reagents such as sodium chloride, chloroform or isopropyl alcohol were not completely removed during RNA extraction (Lucena-Aguilar et al., 2016). The presence of sodium chloride, organic solvents and other compounds will absorb the wavelength at 230 nm, leads to decrease value to the absorbance ratio (Decruyenaere et al., 2023).

Table 2: Concentration and quality of RNA

| Sample	Concentration (ng/ μL	$A_{260/280}$	$A_{260/230}$
Control	654.6	2.105	1.750
Treated (wounded)	758.45	2.090	1.655
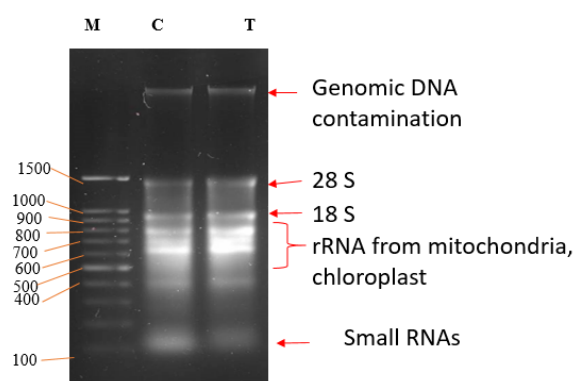
Figure 1   Gel electrophoresis using 1% (w/v) agarose gel. Lane M represents the 100 bp DNA ladder (1st Base). C and T represent control and treated samples respectively.

RNA integrity was analysed using 1% agarose gel electrophoresis to check the presence of ribosomal RNA (rRNA) and RNA integrity in sample. Based on Figure 1, there are several bands exhibited, the number of bands and band size is similar for both control and treated samples. The band size is distinct and not smeared showed RNA is not degraded. The first band on both lanes had the size more than 1500 bp indicates presence of genomic DNA contamination. Genomic DNA has large molecular size so it will appear at the top of gel (Decruyenaere et al., 2023).

The second and third bands near 1500 bp and 1000 bp represent ribosomal RNA 28 S and 18 S respectively. The ribosomal RNA in the sample represents as large subunit of rRNA; 28 S and small subunit rRNA; 18 S (Parker, Balasubramaniam, Sallee, & Parker, 2018). Besides that, common technique in assessing the intact RNA is the intensity ratio of rRNA 28S to 18S must be 2: 1 (Skrypina et al., 2003).

The following band with size of approximately 800 bp, 700 bp and 600 bp may be rRNA from chloroplast or mitochondria. A lightly smeared band occurred around size of 100 bp represents small RNAs such as 5S RNA and tRNAs (Ahlberg et al., 2021). The presence of genomic DNA is due to it not being digested after RNA extraction (Li et al., 2022). Genomic DNA contamination can be eliminated by performing DNase treatment whereby DNase enzyme digest DNA in RNA sample (Hashemipetroudi et al., 2018).

M 1 2 3 4 5

300 —
200 —
150 —
75 —
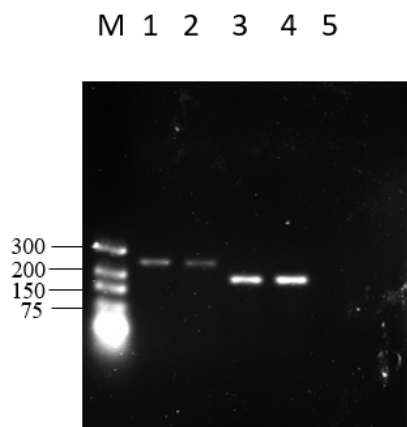
Figure 2   Gel electrophoresis using designed primer pairs. PCR products using tubulin for control sample (lane 1) and treated sample (lane 2), PCR products using HMGR for control sample (lane 3) and treated sample (lane 4), negative control (lane 5). Lane M represents the GeneRuler Ultra Low Range DNA Ladder (Thermo Scientific).

The detection of HMGR gene in control and treated *P. odorata* was performed with RT-PCR using designed HMGR primers. Based on Figure 2, there was a bright, single band on lane 1 and 2 with the size around 200-300 bp, while size of HMGR is about 200 bp shown on lane 3 and 4. Hence, HMGR and tubulin gene were presented for both control and treated *P. odorata*. HMGR gene has higher band intensity than that of its reference gene, tubulin in both control and treated samples. Thus, HMGR was highly expressed in both control and treated plants. Tubulin was selected as the reference gene because it was a housekeeping gene. It is highly expressed in various plant tissues, developmental stage and commonly used in most plants (Yu et al., 2019). Another factor tubulin has stable expression. Dong et al. (2011) reported that tubulin is stably expressed in five tissues; leaves, flowers, fruits, stems and roots.

Table 3: Measurement of area, percent for reference gene and HMGR in control and treated samples and its normalization

		Sample	Area	Percent	Normalization
Reference gene; tubulin	1	Control	1252.799	12.735	
	2	Treated	708.385	7.201	
HMGR	3	Control	3707.577	37.689	2.9594
	4	Treated	4168.577	42.375	5.8846

To further analyze the gel electrophoresis results, the gel image was analyzed using image J tools. The measurement of result was tabulated in Table 3. The area of graph corresponds to its band intensity and gene expression. Based on the result, HMGR has a higher peak area and percentage than that of reference gene; tubulin. This indicates HMGR has higher expression because HMGR is the enzyme responsible in catalyzing reaction for the rate-limiting step for mevalonate pathway in terpenoid biosynthesis. In control or untreated *P. odorata*, the peak area for HMGR is 3707.577 (37.689%) while the peak area for tubulin is 1252.799 (12.735%). For wounded or treated *P. odorata,* the peak area for HMGR has a significantly larger peak area with 4168.577 (42.375%) while tubulin has lowest peak area of 708.385 (7.201%).

Normalization is important in RT-PCR analysis to get more reliable data by normalize band intensity of target gene to internal standard or expressed reference gene (Guenin et al., 2009). The expression of

HMGR gene was normalized with expressed tubulin for both control and treated sample. Therefore, the ratio value of intensity was obtained. HMGR expression was recorded with ratio 2.9594 in control sample and increased to 5.8846 in treated sample. Hence, HMGR has higher expression in *P. odorata* under wound treatment as compared to control or untreated *P. odorata.* This result correlates with findings from other research whereby abiotic stress upregulates HMGR expression in *Ginkgo biloba* (Rao et al., 2019). Besides, wounding may cause an increase in terpenoid content which aids in plant defence mechanism.
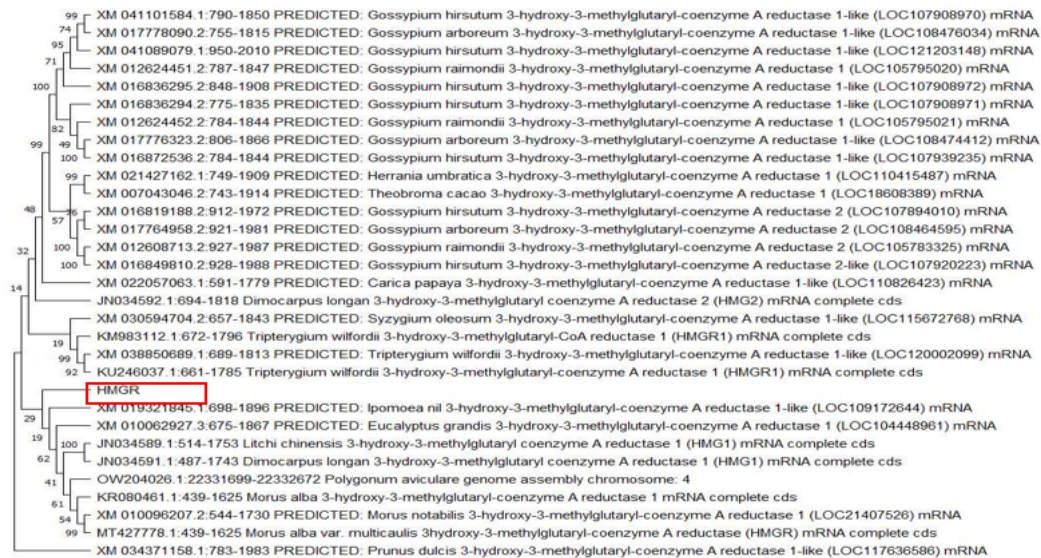
Figure 3:   The Maximum Likelihood tree of HMGR in *Persicaria sp.* and 30 other species with related HMGR sequence. *Prunus dulcis* HMGR served as an outgroup. The stability of each nodes was tested by bootstrap analysis with 1000 replicates. The number below branches are bootstrap value.

Phylogenetic tree was performed to predict the evolutionary relationships of HMGR in *Persicaria* and 30 other species with related HMGR sequence. Gene evolution occur under biotic and abiotic stresses, tissue specific and growth development (Yun et al., 2022). These factors lead to gene speciation which has similar function under same selection pressure across related species and also gene duplication whereby new gene function acquired under different selection pressure within the same or related organism (Young & Gillung, 2019).

The HMGR sequence of *P. odorata* was retrieved from NCBI database and 30   related sequences were selected to perform multiple sequence alignment using ClustalW followed by phylogenetic tree using Mega software. The bootstrap test of 1000 bootstrap replications was used for phylogeny test. The bootstrap test is a resampling test whereby replicate alignment is generated for each cycle to produce a replicate tree (Russo & Selvatti, 2018). The bootstrap value in the generated tree indicates the significance of the support or probability of 'real' clade (Russo & Selvatti, 2018). A bootstrap value with threshold 70% or more shows good support (Park et al., 2010).

Based on Figure 4.6, the 31 HMGR sequence are classified into two groups; group 1 with 14% bootstrap value and group 2 with 29% bootstrap value. This shows evolution and divergence of HMGR. Group 1 can be divided into 2 sub clades, whereby the first sub clade was extensively divided further. It mainly consists of genus *Gossypium; Gossypium hirsutum, Gossypium arboretum, Gossypium raimondii* and also *Herrania umbratica Theobroma cacao. Carica papaya, Dimocarpus longan*. The second sub clade with bootstrap value 19 from group 1 are divided into *Syzygium oleosum* and *Tripterygium wilfordii*.

The second group includes HMGR from *Persicaria*. The finding shows that *Ipomoea nil* has the closest relationship with HMGR from persicaria, with 3 nodes apart and significant value of 19. Although the phylogenetic tree shows a close relationship, but it is insignificant due to low bootstrap value.

Another sub clade was furtherly divided into few sub clades and evolved. At the bottom of the phylogenetic tree, *Prunus dulcis* appear to be as outgroup as it diverged before undergoes division and speciation. This shows that *Prunus dulcis* is more distantly-related and has least related to HMGR from the other species and *Persicaria* (Jamil, 2019).

Phylogenetic tree relationship also can be inferred based on the nodes which shows speciation or duplication. Ortholog share common ancestor due to speciation, while paralog share common ancestor due to duplication (Stamboulian et al., 2020). The orthologous relationship indicates the species retain similar molecular and biological function while the later relationship indicates the divergence of genes which leads to acquiring new or different function (Altenhoff, 2019). *Litchi chinensis* HMG1 and *Dimocarpus longan* HMG1 had an orthologous relationship with 100 bootstrap values. This indicates the similar function of HMG1 in these two species. HMGR1 and HMGR2 from *Dimocarpus longan* exhibited a paralogous relationship. Though they share similar sequence and domain, however they have slightly different function and regulation in HMGR reaction such as tissue specific and plant development. Research also found that HMGR1 has higher expression and is involved in early stage of fruit development, while HMGR2 has higher expression in seed growth in *Dimocarpus* longan (Xia et al., 2011). Besides that, the duplication into group 1 and 2 shows the paralogous relationship between the group of species. In addition, *Gossypium hirsutum, Gossypium arboretum*, *Gossypeum raimondii, Tripterygium wilfordii* has more gene duplications as compared to other species. This shows the potential of these gene evolution for regulatory roles in specific plants.

Conclusion

In conclusion, RNA from both samples was successfully extracted with concentration of 654.6 ng/ μL and 758.45ng/ μL for control and treated sample respectively. The RNA sample has low protein contamination based on absorbance of 260/280 but absorbance 260/230 shows that both sample contain salt and organic solvent. Control and treated samples have good RNA integrity due to presence of 28S and 18S rRNA. HMGR expression value for wounded *Persicaria odorata* (5.88) is higher when compared to untreated *Persicaria odorata* (2.96). Biotic stress using wounding method is able to upregulate the HMGR expression in *Persicaria odorata* to produce higher content of terpenoids. Phylogenetic analysis revealed that HMGR in *Ipomoea nil* has the closest relationship with HMGR in *Persicaria.* The research showed the importance of HMGR gene expression in wounding treatment. Future work may further analyze the effect of wounding treatment in HMGR gene expression quantitatively using real-time PCR. Besides that, terpenoid content in the plant can be measured to validate the finding in this research. By knowing the significance of HMGR in *Persicaria odorata*, HMGR gene can be manipulated and cloned through genetic engineering techniques for better terpenoid production.

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