Trop J Nat Prod Res, November 2023; 7(11):5221-5225
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Identification of the Geranylgeranyl Pyrophosphate Synthase (GGPS) Gene Family in Teak (*Tectona grandis*)

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ARTICLE INFO

Article history:
Received 10 September 2023
Revised 17 October 2023
Accepted 02 November2023
Published online 01 December 2023

ABSTRACT

Teak (*Tectona grandis*, L.f.) plants are known to have the best quality wood in the *Lamiaceae* family but are also known to contain bioactive compounds that are important for pharmacology, both cosmetics and medicines. One of the phytochemical compounds contained in teak is retinol, which is important in cosmetic technology as an antiaging agent. Retinol is a derivative of carotene and carotene is a derivative of terpenoids. At the gene level, in model plants, the mechanism of terpenoid synthesis is controlled by a family of genes known as Geranylgeranyl Pyrophosphate Synthase (GGPS). The basic science of the GGPS gene is important to develop retinol production in teak. This research aims to identify the GGPS gene family in teak plants and observe the expression profile of two GGPS genes in teak plants, GGPS3 and GGPS6. The approach taken in this study used *de novo* NGS-transcriptome and qRT-PCR analysis. The results of this research were the results of transcriptome analysis, there were 87,365 contigs and seven GGPS gene sequences were identified. GGPS3 and GGPS6 were tested for expression profiles in young and old leaves using qRT-PCR. There is an up and down-regulation mechanism of the GGPS3 and GGPS6 genes in old and young leaves, indicating a feedback mechanism for these two genes. These results provide a strong basis for engineering terpenoid and retinol synthesis in teak plants.

Keywords: Tectona grandis, terpenoid, NGS, qRT-PCR, DDGS

Introduction

Teak (*Tectona grandis*) is a woody plant member of the *Lamiaceae* family. Teak is well known for the quality of the wood that is strong and resistant to pests, especially termites. Teak has rapid growth during the vegetative phase and is very slow after entering the generative period.[1,2,3] It can be found in several regions of South Asian countries and its parts such as root, bark, flowers, wood, and oil are reported to be an important source of medical properties. The various parts of the plant have been used traditionally and ethnopharmacologically for the treatment of common cold, headache, wound healing, bronchitis scabies, as a laxative, diuretic, antidiabetic, anti-inflammatory, antioxidant, lipid disorders, constipation, and diuretic.[4,5,6] This pharmacological activity was found to come from secondary metabolites from the terpenoid group.[7,8]

Terpenoids represent a prominent class of metabolites present in all living organisms.[9] A vast and structurally diverse number of terpenoids is produced by plants, which employ multiple biosynthetic pathways frequently acting in parallel to create a plethora of these metabolites[10]. Some terpenoids found in all plants serve essential roles and include pigments (chlorophylls, carotenoids), electron carriers (plastoquinone, ubiquinone), membrane components (sterols), and hormones (gibberellins, abscisic acid, steroids, strigolactones).[9,10]

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Citation: Diningrat DS, Marwani E, Kusdianti Identification of the Geranylgeranyl Pyrophosphate Synthase (GGPS) Gene Family in Teak (*Tectona grandis*). Trop J Nat Prod Res. 2023; 7(11):5221-5225. http://www.doi.org/10.26538/tjnpr/v7i11.28

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

In contrast, most of the plant terpenoids, mono, and di-terpenes, are species-specific specialized metabolites that are involved in antagonistic and beneficial interactions with the environment.[10]

All terpenoids are derived from the basic unit structure of five carbon atoms: isopentenyl pyrophosphate (IPP) and its allyl isomer dimethyl allyl pyrophosphate (DMAPP).[11] In the plastids of plants, IPP and DMAPP are synthesized by the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway.[12] Three molecules of IPP and one molecule of DMAPP form the 20-carbon compound geranylgeranyl pyrophosphate (GGPP) in the action of GGPS. Geranylgeranyl pyrophosphate synthase (GGPS) is a structural enzyme in the terpene biosynthesis pathway and a member of the isopentenyl pyrophosphate synthase gene family.[11,13]

In recent years, some progress has been made in the functional research of GGPS genes. The physiological and biochemical functions of GGPS are closely related to its tissue expression characteristics and subcellular localization.[14] There are 12 GGPS genes in the *Arabidopsis thaliana* genome[11]. There has been no previous research related to this in teak plants, therefore it is assumed that it will be the same as the model plant.

The rapid development of molecular biology has resulted in new technologies in the analysis of expression patterns, namely the Next Generation Sequencing (NGS)-Transcriptome Analysis approach. NGS-transcriptome analysis has made it possible to analyze the expression profiles using short sequences.[15,16] The NGS approach can result in what is called the Differentially Gene Expressed (DEG) of interest gene at each stage.[15] The DEG data can be validated *in silico* on the target plant tissue by qRT-PCR analysis approach.[15,16] QRT-PCR analysis can quantify the expression of the gene of interest as a confirmation of the results of DEG analysis.[15,17] The aim of this research is as a first step to identify the GGPS gene in teak plants.

Materials and Methods

Teak tissue materials and RNA isolation

Young and old leaves of teak (Figure 1) were collected from a 22-year-old teak plant at the Institute of Technology Bandung, Indonesia for RNA isolation in April 2023. Sample collection *Tectona grandis* (coll. no. Diningrat 2001; ITB campus Jl. Ganesha, Bandung District, West Java Province; the coordinates of location at -6.887664346483448, 107.60932232621624) and plant sample was submitted to the Herbarium Bandungense, Institute Technology Bandung of Indonesia, Bandung for its identification by Arifin Surya Dwipa, PhD., and the voucher specimen was 3689/II.CO2.2/PL/2022. The following vegetative tissues were sampled from young and old leaves. Both teak tissue samples were frozen in liquid nitrogen immediately upon collection. Samples were immediately frozen at -80. Total RNA was obtained using the method for RNA isolation protocol that was developed by Chang *et al* (1993) [1,15]. Frozen tissue was ground to a fine powder under liquid nitrogen and dispersed in a CTAB buffer. Following 2 chloroform extractions, RNA was precipitated with $LiCI_2$, again extracted with chloroform, and precipitated with ethanol. The resulting RNA pellet was resuspended in 20-100 µl of DEPC-treated water. RNA concentration analysis was conducted on a QubitTM fluorometer (www.invitrogen.com/qubit) to show the total yield of the RNA sample. The integrity of RNA was assessed with the Agilent 6000 RNA Nano Chip Kit on 2100 Bioanalyzer (Agilent Technologies).[18]

Paired-end cDNA library preparation and MiSeq Illumina sequencing

The total RNA of the teak was extracted from the two tissues using the protocol described previously. The double-stranded cDNA was synthesized using the cDNA Synthesis System using random hexamer primers (Illumina) according to the manufacturer's instructions.[19] The paired-end library was developed according to the protocol of the Paired-End Sample Preparation kit (Illumina, USA).[15,19] The resulting library was sequenced at Penn State University using Illumina MiSeqTM 2000 (Illumina Inc., USA).

Transcript assembly and annotation

Two sequence data in FASTQ files computed with CLCbio for transcript assembly strategy, paired-end reads were trimmed for quality score and the presence of repeated sequences>50 bp using the modified Mott-trimming algorithm present (default parameters) in CLCbio[14]. Contig assembled *de novo* the Illumina-trimmed paired-end reads into transcript contigs using the software CLC Genomics Workbench by setting a minimum 95% identity, minimum 40% overlap, and 200 bp as minimum contig length. The quality of the *de novo* assembly was assessed with a local BLASTN (e-value< 10^{-6}) alignment of all the contigs against *S. lycopersicum* (www.phytozome.com) using CLCbio workbench[1,15]. After teak GGPS sequences were obtained, phylogenetic analysis was performed on the GGPS sequences to determine the GGPS gene diversity that exists in teak. Phylogenetic analysis was also conducted to determine the teak GGPS position compared with the GGPS of the other plant using the BLASTX analysis approach of the NCBI nr-protein database. Phylogenetic analysis is performed using the MrBayes: Bayesian Inference of Phylogeny (http://mrbayes.sourceforge.net/).[1,20]

Analysis of DEGseq

Comparison of differential gene expression (DEGseq) between GGPS in young and old leaves was done using RNAseq analysis software developed by CLCbio genomic work bench[1,15]. The DEGseq analysis was used to identify GGPS genes in transcript abundance because it integrates several statistical methods.[21] The number of reads per contig for each GGPS gene was compared between young and old leaf tissues in teak separately. RNAseq employs a random sampling model based on the read count in young and old leaf tissue libraries and performs a hypothesis test based on that model. Further analysis of the DEG results should be validated by qRT-PCR.[1,17,21]

Validation Tests of GGPS By Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR tests were conducted to determine the extent to which the number of EST reads per gene obtained by shotgun sequencing accurately reflected transcript levels in the source tissues.[22,23] RT-PCR estimates of transcript abundance were conducted on RNA from young and old leaf tissues from teak. Quantitative real-time PCRs (qRT-PCRs) were prepared using the SYBR Green Master Mix kit (Applied Biosystems) and run in an Applied Biorad CFX 96 Fast Real-Time PCR system with default parameters. Primers were designed using Primer3 software. The parameters used were the default parameters of Primer3. The parameters were set as follows number to return = 5, max stability = 9, max repeat mis priming = 12, pair max repeat mis priming = 24, max template mis priming = 12, and pair max template mis priming = 24. Parameters for thermodynamics also use the default parameters consisting of primer size optimum = 20 (18-27), primer Tm optimum = 60 (57-63), max Tm difference = 100, % primer GC minimum 20, and maximum 80. (http://bioinfo.ut.ee/primer3-0.4.0/input-help.htm)[1]. A gene encoding 18S rRNA was used as an endogenous standard to normalize template quantity. QRT-PCR analyses were performed to confirm the expression of GGPS using *in silico* expression analysis[1,18]. For each GGPS gene, three biological replicates and three technical replicates were performed. Statistical analyses were used to estimate the significance of the differences.[16,17] For the analysis of gene expression of GGPS in the vegetative to generative transition of teak, primers of GGPS3 and GGPS6 were designed using Primer3 software. The primer sequence of GGPS3 is Left Primer (L): CACCCGTTCATTGTGACTGA, Right Primer (R): GCATTTTGGGCTTGTTGATA, L: CATTTGCTTTGTTGCCTCAC, R: TGCTCTGCCCACTTTTGTAG for GGPS6 and L: AATTGTTGGTCTTCAACGAGGAA, R: AAAGGGCAGGGACGTAGTCAA for 18S.

(a) (b)

Figure 1: Teak leaf samples: (a) Young teak leaf; (b) old teak leaf

Result and Discussion

Next-generation sequencing (NGS) transcriptome analysis of young and old teak leave

RNA was isolated from the teak tissue of vegetative and generative shoot buds. RNA isolation was carried out using a modified method from Chang *et al*, (1993).[1,15] The RNA concentrations are 555 and 206 ng/µl for vegetative and generative samples, respectively. The quality of RNA was checked with qubits and bioanalyzer, and only RNA with the best RIN values was further analyzed using the Illumina NGS-Miseq platform.[1,15] RNA from teak young leaves had a 7.7 RIN value and 8.2 for teak old leaves. Sequencing using the Illumina Miseq platform generated as many as 3,701,878 and 3,778,316 sequences for young and old leaves, respectively. These sequences were further analyzed, with trimming analysis using a CLC bio workbench to determine the quality of the sequence.[18,19] The trimming results showed that the sequence has good quality. The following analysis is *de novo* assembly using a CLC bio workbench.[15,23] The results of the *de novo* assembly were 87.365 contigs. It resulted from the combination of vegetative and generative tissue sequences. Contig quality was also tested by trimming using a CLCbio workbench.[16] The trimming of the contig results can be seen in Table 1.

Then, contigs are annotated with *S. lycopersicum*. The results from BLASTN and annotations of teak contigs against *S. lycopersicum* cds database, which produced 7 contig hits clicking GGPS gene. All

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

GGPS contig then it calls GGPS unigene. The BLASTN results are presented in Table 2. The GGPS unigene had different E-values and % identity. In this study decided to choose GGPS3 and GGPS6 for further analysis because it has the lowest E-value. GGPS3 and GGPS6 unigenes were then further analyzed by phylogenetic analysis using MrBayes: Bayesian Inference of Phylogeny (http://mrbayes.sourceforge.net/).

Geranylgeranyl Pyrophosphate Synthase (GGPS) in teak

The results of phylogenetic analysis showed that there were two major groups of GGPS in teak. It can classify into 2 major groups of genes GGPS namely: major group I is a group of GGPS from Lamiales. In this group, there are several genes teak GGPS, teak GGPS7, teak GGPS4, and teak GGPS2. Teak GGPS are in this group, it can be presumed that teak GGPS genes are identical to the GGPS gene of Lamiales; Major group II consists of eight genes GGPS of *Populus trichocarpa*, GGPS of *Eucalyptus grandis*, teak GGPS3, teak GGPS5, LHY (Hypocotyl Elongation gene) of *Arabidopsis thaliana* (2nd out group), teak GGPS1, GGPS of *Oryza sativa* and teak GGPS6 (Figure 2). Major group II contained GGPS genes that are not derived from Lamiales. This second group consists of precisely GGPS genes of Poplar and Eucalyptus, The LHY gene of Arabidopsis is a non-GGPS outgroup as a comparison, as well as a comparison of GGPS monocot *Oryza sativa*.

Table 1: Statistical summary of sequencing and *de novo* assembly results

Parameter	Value
Input sequence	3,701,878 and 3,778,316
Total bases	42,435,728
Contigs	87,365
Minimum length of contigs	225
Maximum length of contigs	4,361
The average length of contigs	486

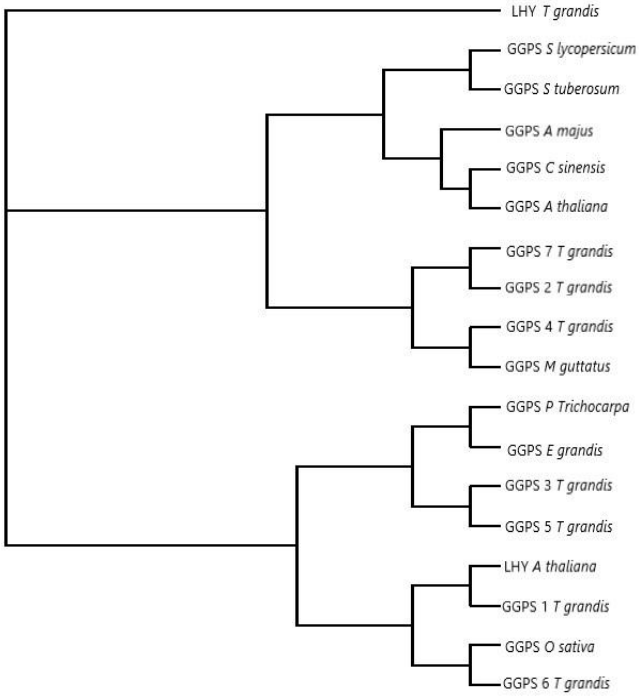

Figure 2: Phylogenetic tree of teak GGPS genes compared with GGPS gene from the other species from gene bank database (http://ncbi.nlm.nih.gov/)

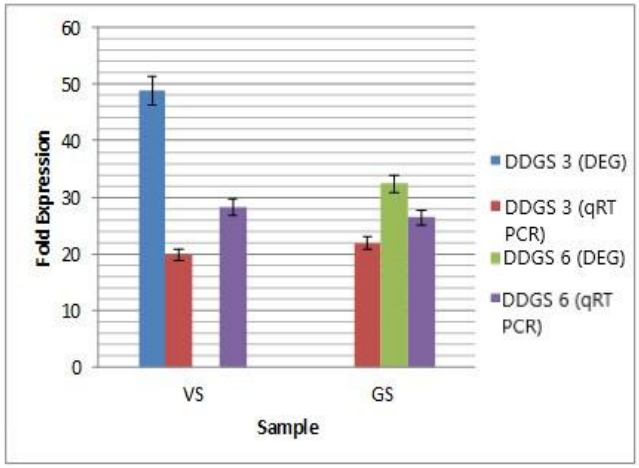

Figure 3: Expression level GGPS3 and GGPS6 gene in the regulation of generative organ formation from young leaves (VS) to old leaves (GS).

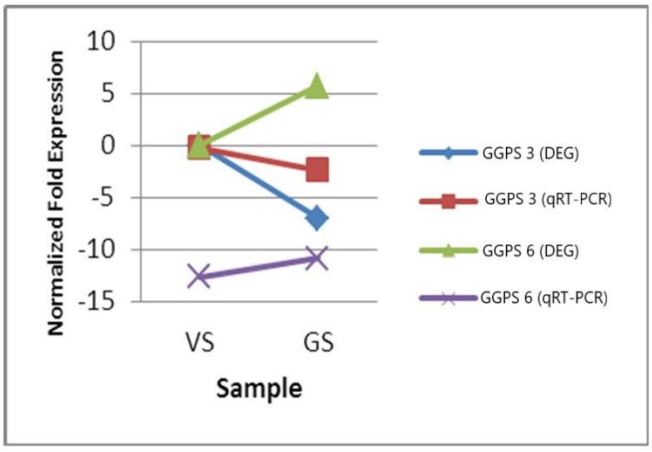

Figure 4: Expression profiles of GGPS3 and GGPS6 genes in the regulation of generative organ formation from young leaves (VS) to old leaves (GS).

Teak GGPS existing in major group II can be thought to be a typical GGPS gene in teak. For this reason, teak GGPS3 and GGPS6 were chosen for further analysis. Another reason why chose GGPS3 and GGPS6 is because it has the smallest E-value resulting in BLASTN analysis with the S. *lycopersicum* cds database (www.phytozome.com).

The GGPS3 and GGPS6 expression profiles in teak leaves

The results of the DEG and qRT-PCR analysis of GGPS3 and GGPS6 gene-level of expression can be seen in Figure 3. These results of DEG need to be confirmed in the vegetative and generative tissues of teak with qRT-PCR analysis. The results of the expression profile of qRT-PCR analysis of gene GGPS3 and GGPS6 in the generative and vegetative tissue of teak shows that the GGPS3 and GGPS6 gene expression profile results of qRT PCR are equivalent to the expression profile results of DEG analysis (Figure 4). GGPS3 expression profiles in downregulated as age increases, young leaves become old leaves. GGPS6 expression profile is opposite to GGPS3 expression profiles in the change of young leaves to old leaves, GGPS6 in up-regulation of genes.

Teak is a plant that has a high economic value and reasonably gains attention for quality and quantity improvement.[1,3] The content of anthocyanins which are terpenoid derivatives in plants is different in young and old leaves. This is the basis for research on teak plants. One of the regulations that regulate this is the activity of the GGPS gene. This GGPS expression profile research on teak is expected to provide

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

additional information on the mechanism of terpenoid production. In this study, the approach used NGS-transcriptome analysis to identify GGPS genes in teak.[15,17,18] The results of NGS-transcriptome analysis of the teak sequences obtained seven kinds of GGPS unigene, which is annotated with *S. lycopersicum*[1,15].

In the *Arabidopsis* model plant, 12 GGPS alleles have been identified (ABRC) (www.arabidopsis.org).[10] Although each allele has its expression profile, the general profile of GGPS expression is downstream which determines the direction of young organs into old organs. In the model plant, terpenoid production is induced by environmental and endogenous cues that enable high-level expression of GGPS in the flank of shoot apical meristem; therefore, floral meristem and subsequently, floral primordium get produced[11,12]. GGPS protein directly promotes the expression of the transcription factor IPP and DMAPP. IPP and DMAPP are subsequently utilized by short-chain prenyltransferases that join the isoprene units in the trans configuration to form larger prenyl diphosphate intermediates, which ultimately serve as precursors for the downstream terpenoid biosynthesis. Farnesyl diphosphate synthase (FPPS) forms trans farnesyl diphosphate (E, E-FPP) in the cytosol, while geranyl diphosphate synthase (GPPS) and geranylgeranyl diphosphate synthase (GGPPS) are responsible for respective geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) formation primarily in plastids[10,11].

In teak, terpenoid production was also induced by environmental and endogenous factors. [7,8,24] In the model plant which is induced GGPS gene as a structural enzyme in the terpenoid biosynthesis pathway.[12,13,14,25] In this study, the expression of two of the seven teak GGPS, namely the GGPS3 and GGPS6 genes have an expression pattern of up and down-regulation as part of the terpenoid biosynthesis process in young and old leaves.

The results of DEG and qRT PCR gene expression analysis showed that GGPS6 maintained the up-regulation trend in the regulation of terpenoid formation. These results indicate that the GGPS6 expression profiles were equivalent to the general pattern of GGPS expression in the model plant. GGPS3 expression profiles are not equivalent to the general pattern of GGPS expression profiles in the model plant. Based on the results it can be assumed that teak have more than one kind of GGPS. We need further analysis to identify other GGPS unigene existing in the teak EST database that resulted from NGS-transcriptome analysis were performed. To further identify GGPS genes in teak, requires advanced gene expression analysis, including in situ hybridization, gene over-expression, and gene silencing.

However, this result is an initial study of GGPS, one of the structural enzymes in the terpene biosynthesis pathway of gene expression in the teak terpenoid regulation. The results of this study may provide a basis for further research in understanding the regulatory mechanisms of terpenoid production in teak.

Conclusion

This research provides *de novo* assembly results from NGS-Transcriptome analysis of young and old teak leaf sequences which produced 87,365 contigs. Identification and annotation results with the *S. lycopersicum* CDS database obtained results of seven different unigene GGPS in teak. GGPS3 and GGPS6, which have the smallest value of the E-value, which is the same major group were analyzed further by DEG analysis and qRT-PCR analysis. DEG expression profile results of GGPS3 and GGPS6 in equivalent to qRT-PCR results from both genes. GGPS6 has a profile that is equivalent to the general GGPS expression profile in the model plant. While GGPS3 has the opposite profile compared with the general profile GGPS. Further research is needed to develop an understanding of the GGPS gene in teak, both the profile of the GGPS gene and its expression patterns related to terpenoid biosynthesis in various organs of the teak plant. However, even though in this study only two GGPS genes were tested for their expression profiles, the results of this study have provided an important basis regarding the GGPS expression profile in the mechanism of terpenoid production in teak leaf organs.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them

Table 2: Contigs-related GGPS genes result from BLASTN to the *S. lycopersicum* CDS database (www.phytozome.com)

Query	Gene Name	Number of hits	Lowest *E-value*	Accession (*E-value*)	Description (*E-value*)	Greatest identity %	Greatest hit length	Greatest bit score
Teak-D-LB2_12_L001_R1_001 (paired) contig 30107	*GGPS1*	170	0.026	Solyc03g118160.1.1	PACid:27291183	100	26	38.158
Teak-D-LB2_12_L001_R1_001 (paired) contig 44286	*GGPS*2	51	0.009608	Solyc03g118160.1.1	PACid:27291183	100	20	40.140
Teak-D-LB2_12_L001_R1_001 (paired) contig 45759	*GGPS*3	124	1.829E-39	Solyc03g118160.1.1	PACid:27291183	100	290	163.046
Teak-D-LB2_12_L001_R1_001 (paired) contig 51758	*GGPS*4	28	0.515	Solyc03g118160.1.1	PACid:27291183	100	21	34.193
Teak-D-LB2_12_L001_R1_001 (paired) contig 54733	*GGPS*5	29	0.472	Solyc03g118160.1.1	PACid:27291183	100	17	34.193
Teak-D-LB2_12_L001_R1_001 (paired) contig 56427	*GGPS*6	39	7.581E-12	Solyc03g118160.1.1	PACid:27291183	100	179	69.875
Teak-D-LB2_12_L001_R1_001 (paired) contig 87272	*GGPS7*	36	0.14	Solyc03g118160.1.1	PACid:27291183	100	21	36.175

Acknowledgments

This research project was financially supported by the Directorate General of Higher Education, Research and Technology Ministry of Education, Culture, Research, and Technology through basic domestic cooperation research with no contract 019/UN33.8/DRTPM/PL/2023. We appreciate the facilities provided by the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan, The School of Life Sciences and Technology, Institute Technology Bandung and Department of Biology, Faculty of Mathematics and Natural Sciences Education, Universitas Pendidikan Indonesia for this work.

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