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EXPRESSION ANALYSIS OF ANTHOCYANIN BIOSYNTHETIC GENES FROM THE YOUNG AND MATURE LEAVES OF *TECTONA GRANDIS* L.F.

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ABSTRACT

Tectona grandis L.f. is a tropical hardwood tree which is known for its timber yield. Apart from its durable wood, the leaves of teak are rich source of anthocyanin. The main objective of the present study was to analyze the expression profile of major anthocyanin biosynthetic genes from the young and mature leaves of teak. The young leaves of teak contains significant amount of anthocyanin when compared to mature leaves. The key enzyme in the anthocyanin biosynthetic pathway, PAL, the regulatory enzyme, CHI and the end point enzyme UGT showed sound activity in young leaves. The obtained results were further correlated by the higher expression profile of major anthocyanin biosynthetic genes such as *CHS1*, *CHS2*, *CHI1*, *CHI2*, *F3'5'H1*, *F3'5'H2*, *DFR*, *ANS* and *UF3GT* in the young leaves.

Keywords: *Tectona grandis* L.f., Anthocyanin, Biosynthetic genes, Phenylalanine ammonia lyase, Chalcone synthase.

1. INTRODUCTION

Anthocyanins are water soluble pigments, a unique class of flavonoids and form one of the largest group of phenolics reported from the plant world. Anthocyanins are polyphenolics responsible for the cyanic colours ranging from salmon pink through red and violet to dark blue of flowers, fruits, leaves, stems and are reported from all the higher plant groups. The flavonoid biosynthetic pathway is the basis for the wide shades of anthocyanin reported in the nature. The precursor molecule, phenylalanine marks the branching point of primary and secondary metabolite synthetic pathway from which the phenylpropanoid metabolism (PPM) initiates. The phenylalanine is transformed into trans-cinnamic acid by the enzyme phenylalanine ammonia lyase(PAL) and is regulated by the PAL encoding specific genes [1]. Condensation of trans-cinnamic acid results into the formation of 4-coumarate coenzyme A which in turn reacts with three molecules of malonyl Co A and generates naringeninchalcone (the enzyme was chalcone synthase, CHS). Naringeninchalconeis catalyzed by different group of enzymes leads to the formation of diverse flavonoid groups i.e., anthocyanin or proanthocyanidin or other polyphenolic compounds. Generally, the expression of *CHS* genes at transcriptional and translational level is low but initiates mass production due to their higher turnover number. The naringeninchalcone is converted into its isomeric form naringeninflavonone i.e., with three rings of basic flavonoid skeleton (C6-C3-C6). The enzyme flavanone-3-hydroxylase(F3H)modify naringenin to dihydro-kaempferol, which inturn into dihydroquecertin and dihydromyricetin by the enzymes flavonoid 3' hydroxylase (F3'H) and flavonoid 3'5'hydroxylse (F3'5'H) respectively. In addition, dihydroflavonol 4-reductase (DFR) reduces these dihydroflavonols to their respective leucoanthocyanidins. Anthocyanidin are formed from the oxidation of its immediate colourless precursor leucoanthocyanin with the help of ferrous ion and is catalyzed by the enzyme anthocyanidin synthase (ANS). This is further subjected to glycosylation by UDP glucose: flavonoid-O- glycosyltransferase (UFGT). The enzymes involved in this pathway are localized in the cytosol and after the synthesis of anthocyanin they are compartmentalized either into the vacuole or cell walls.

The structural genes encoding the enzymes in anthocyanin synthesis are conserved in the plant kingdom. Many studies revealed that the expression of genes in the anthocyanin synthesis was positively correlated with the anthocyanin content [2]. Among the structural genes, *PAL* is the first gene associated with

anthocyanin synthesis. Varied views were reported regarding the *PAL* gene expression i.e., anthocyanin was produced at the starting point with the level of conversion of phenylalanine by the active phase of the PAL enzyme. Meanwhile, others suggest that the precursor for anthocyanin was sufficient for its synthesis andtherefore not depend on the PAL activity. The genes encoding for chalcone synthase (CHS), the marker enzyme which catalyze the formation of naringeninchalcone constitute a multigene family and was the best studied plant-specific type III ployketide synthase. *CHS* gene has been isolated and well characterized in many plant species, especially in eudicots [3]. In this scenario, the present study aims to analyze the expression profile of some anthocyanin biosynthetic genes from the young and matured leaves of teak.

2. MATERIAL AND METHODS

2.1. Plant material

Teak (*Tectona grandis* Lf.) was collected from the natural habitat and authenticated by comparing with the document of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala. The part of the teak selected for the study was coloured young leaves (collected from the first node at the apex of the shoot) and green mature leaves (collected at the fourth node from the apex of the shoot).

2.2. Quantification of anthocyanin

The monomeric anthocyanin content from young and mature leaves was quantified by the pH differential method [4].

2.3. Isolation of phenyl alanine ammonialyase (PAL), chalconeisomerase (CHI) and UDP-glycosyltransferase (UGT) in the young and matured teak leaves

Fresh young and mature leaf samples (1 g) were pulverized into fine powder using liquid N_2, and subsequently homogenized using 5 mL Na_2HPO_4/ KH_2PO_4 buffer (pH 7.0) containing 5 mmol/L β-mercaptoethanol, 5% (w/v) polyvinylpolypyrrolidine (PVPP), 50 mmol/L ascorbate and 0.05% (v/v) Triton X-100. The macerate was centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was used as the enzyme source to analyze the activities of phenylalanine ammonialyase (PAL), chalconeisomerase (CHI) and UDP-glycosyltransferase (UGT). The PAL activity was measured by analyzing the synthesis of trans-cinnamate from deamination of phenylalanine. One PAL unit activity (U) was referred as an increase of 0.01 in absorbance at 290 nm. CHI activity was evaluated using the chalcone degradation at381 nm, 34C. One unit activity (U) of CHI was the reduction of 0.01 in absorbanceat 381 nm /h. UGT activity was measured using the reaction of quercetin with UDP galactose at 351 nm, 30C. One unit activity (U) of UGT activity was the change of 0.001 in absorbance at 351 nm / h.

2.3.1. Assay of PAL activity

The activity of PAL was estimated by the method of Kelij et al. [5]. The activity was related to the amount of ammonia formed by the action of PAL on the substrate. The initial absorbency was read at 290 nm. The reaction was allowed to proceed for 30 min at room temperature and was stopped by the addition of 0.5mL 10 % Trichloro acetic acid (TCA), then the final absorbance was recorded. The difference between initial and final absorbance was used for the estimation of PAL.

2.3.2. Assay of CHI activity

2'4'4'6'-tetrahydroxychalcone (naringeninchalcone) was produced from naringenin by reacting with 50% KOH followed by acidification and recrystallization from aqueous ethanol. Crude 50-200 µL CHI enzyme was added to 50 mMTris-HC1 (pH 7.4) containing BSA (7.5 mg/mL) and 50 mM KCN (to arrest peroxidase disintegration of the tetrahydroxychalcone) to yield 1 mL volume. The reaction was initiated by adding 5 µL of 1 mg/mL tetrahydroxychalcone in 2-ethoxyethanol (final dose of 18.5 µM) and changes in absorbance was recorded at 381 nm with the cell holder maintained at 30C. Spontaneous isomerization of the chalcone was permitted in the reference cell contained the assay mixture without enzyme. The initial disappearance rate of the chalcone (ΔA381) in the presence of enzyme was used to calculate CHI activity.

2.3.3. Assay of UFGT activity

Assay mixture contained 100 µL UFGT enzyme, 100 µL 50 mM bicine buffer (pH 8.5), quercetin, the substrate (15 µL of 2 mg/mL, final dose 1 mM) and10 µL of 15 mg/mL of 2.5 mM UDP galactose (final dose 2.5mM). Reaction tubes were incubated at 30C for 10 - 30 minutes and the reaction was arrested by the addition of 20% 75 µL TCA in methanol. Further, the resultant was centrifuged at 7000 x g for 7 min. The supernatant

was stored at -5C and quantified by HPLC. The HPLC system consisted of a Waters 600 solvent delivery system with a WISP 712 automatic sample injector and a Waters 490 variable UV detector. The column was a 220 x 4.6 mm Aqua pore RP-18 connected with a 15 x 3.2 mm Aquapore RP-18guard column maintained at 28C. 5 µL samples was injected onto the column and chromatographic traces were noted using the Waters/Dynamic Solutions Maxima program. The mobile solvent system used were (A) 10% (v/v) aqueous aceticacid and (B) acetonitrile. The solvents were pumped isocratically using a solvent mixture of 85 (A): 15 (B), at 1 mL/min flow rate for 10 minutes. The end product, quercetin-3-galactoside, was assayed at 350 nm and eluted at 5 minutes.

2.4. Expression analysis

2.4.1. Prediction of genes and designing of gene-specific primers

The entire teak genome sequence and anthocyanin genes were predicted from Teak bulletin, Department of Biotechnology (http://biit.cs.ut.ee/supplementary/WGSteak) employing BLAST (Basic Local Alignment Search Tool) (ncbi.nlm.nih.gov/ Blast.cgi) and FGENESH (www.softberry.com) gene envision calendar. The accession numbers of genes involved in the pathway were chosen for gene prediction from NCBI database includes potato- HQ659493.1, HQ 659498.1, HQ659496.1, AF449422.1, HQ337900.1, DQ106850.1; *Petunia hybrida*- X04080.1,X14589.1, X60512.1, AF233639.1, AB027454.1; egg plant EU809469.1.After recognition of genes, gene specific primers were depicted for each gene by online available Integrated *DNA* Technologies (Idt) DNA oligoanalyser (http://eu.idtdna.com/analyzer/applications/oligo analyzer) software programme. Oligos were customized from BioServe Biotechnologies India Pvt. Ltd. (www.bioserveindia.com). List of oligos and their features were displayed in the Table 1 & 2.

2.4.2. Isolation of RNA

Young and matured leaf samples were collected from natural teak growing areas. Good quality of total RNA was isolated from 100 mg of each sample by using three different methods discusssed in respective sections.

Table 1: List of oligos used in semi-quantitative PCR assay for validation of predicted anthocyanin pathway genes in teak

Primer ID	Primer Sequence (5' - 3')	Length (bp)	Amplicon (bp)	Tm (C)	GC%
CHS1-rtF	TGC TAT TGA TGG TCA CCT TCG C	22	337	57.5	50
CHS1-rtR	GCC TTC ACC TGT GGT ACT AAG C	22	144	57.6	54.5
CHS2-rtF	TCT AGG TAT ATC TGA CTG GAA CTC TC	26	231	54.5	42.3
CHS2-rtR	TCA AGC CCT TCA CCA GTA GTT CC	23	137	58.7	52.2
CHI1-rtF	GTT CCT TGT GTT GGG AAT GCT G	22	287	56.8	50
CHI1-rtR	GACACATTAGACCGAGTTGACAAG	24	142	55.5	45.8
CHI2-rtF	TCT CTG ACG ATG ATT CAG TTC CTG	24	255	56.2	45.8
CHI2-rtR	GAAACACACTCTCAGAAGCTAGGCT	25	151	58.4	48
F3H-rtF	GTC TCA AGC CAC TTA CAG GGTGAAG	25	292	59.2	52
F3H-rtR	GAGTAAGGTCAGGCTCTGGACAC	23	193	58.5	56.5
F3'5'H1-rtF	AAG AAG CTA GTG CGA GTT GTT ACC	24	244	57	45.8
F3'5'H1-qR	TTA CAT GGA GTA GGT GCA TCA T	22	169	53.6	40.9
F3'5'H2-rtF	AAG AAG CTA GTG CGA GTT GTT ACC	24	244	57	45.8
F3'5'H2-rtR	TTA CAT GGA GTA GGT GCA TCA T	22	169	53.6	40.9
DFR-rtF	GTA TGA GCA CCC CAA GGC AGA G	22	244	59.9	59.1
DFR-rtR	CTT CTG TCG GCA AGT CTC AAT GG	23	183	58	52.2
ANS-rtF	AGT GCT TGT GGT CAG CTT GAG TG	23	236	59.7	52.2
ANS-rtR	AGC AGC AAG TCT TCC ATG CCT C	22	146	59.9	54.5
UF3GT-rtF	AGG AAA GAT CGG TGG TGT ATC TAA G	25	265	5.9	44
UF3GT-rtR	AGA ATC GAG TTC CAT CCA CAA TGC	24	144	57.4	45.8
Actin-rtF	ATGACTCAAATCATGTTTGAGACCTTC	27	288	55.5	37.0
Actin-rtR	ACCTTAATCTTCATGCTGCTTGGAGC	26	192	59.4	46.2

Table 2: List of oligos used in quantitative real time PCR assay for validation of predicted anthocyanin pathway genes in teak

Primer ID	Primer Sequence (5' - 3')	Length (bp)	Amplicon (bp)	Tm (C)	GC%
CHS1-qF	CTG AGC CTA AAG CCC GAA AAA CTT C	25	144	58.3	48
CHS1-qR	GCC TTC ACC TGT GGT ACT AAG C	22	144	57.6	54.5
CHS2-qF	CCT GAG AAA CTT AGG GCT ACA AGA G	25	137	56.7	48
CHS2-qR	TCA AGC CCT TCA CCA GTA GTT CC	23	137	58.7	52.2
CHI1-qF	CAGGAAGTGAGCTATCGTCAGTC	23	142	56.8	52.2
CHI1-qR	GACACATTAGACCGAGTTGACAAG	24	142	55.5	45.8
CHI2-qF	CCTGCAGCAAAGCGTAGTCTTG	22	151	58.7	54.5
CHI2-qR	GAAACACACTCTCAGAAGCTAGGCT	25	151	58.4	48
F3H-qF	GACAAACCACAAGGCTGGATAGGTG	25	193	59.7	52
F3H-qR	GAGTAAGGTCAGGCTCTGGACAC	23	193	58.8	56.5
F3'5'H1-qF	ATG GAC TCT CCT ATC TCA GTC G	22	169	55.1	50
F3'5'H1-qR	TTA CAT GGA GTA GGT GCA TCA T	22	169	53.6	40.9
F3'5'H2-qF	TCT TCT TGT TTA CAT GGA GCA TAG G	25	166	54.8	40
F3'5'H2-qR	GCT TCT TCC ATT GTC TTA AAT GGT G	25	166	54.6	40
DFR-qF	GAT GTG GCT AAG ATG GTG CGA CAG	24	183	59.8	54.2
DFR-qR	CTT CTG TCG GCA AGT CTC AAT GG	23	183	58	52.2
ANS-qF	CCT GCT GAC TAC ATT CCA GCA AC	23	146	58	52.2
ANS-qR	AGC AGC AAG TCT TCC ATG CCT C	22	146	59.9	54.5
UF3GT-qF	CAA CGG AGT CAA GAC TTT ACC TAA G	25	144	55.1	44
UF3GT-qR	AGA ATC GAG TTC CAT CCA CAA TGC	24	144	57.4	45.8
18SrRNA-qF	GCGACGCATCATTCAAATTTC	21	154	53.2	42.9
18SrRNA-qR	TCCGGAATCGAACCCTAATTC	21	155	54.4	47.6

2.4.2.1. Guanidium Isothiocynate (GTC) method

Total RNA from the tender and matured leaves of teak was isolated by GTC method [6]. Initially, the fresh leaves were surface sterilized by using RNAase free water and further, 100 mg of leaf tissue was homogenized in a pre-chilled mortar using liquid nitrogen into fine powder. 500 µL of solution D (4MGTC, 25 mM sodium citrate, 0.5% sarcosine and 0.1M β-mercaptoethanol) was added and the fine powder was made into a slurry. The slurry was then transferred to a clean sterile eppendorf tube (1.5 mL). 100 µL of 2 M sodium acetate pH 4.0 was added to the slurry and mixed thoroughly. Subsequently, 400 µL of citrate saturated phenol (pH 4.3) was added gently and mixed by inversion followed by vortexing for3 min. Subsequently, 200 µL of chloroform:isoamyl alcohol was added, and vortexed for 5 min. The resulting mixture was incubated on ice for 15 min and centrifuged at10000 g for 10 min at 4°C. The upper aqueous layer containing RNA was carefullytransferred to a sterile vial without mixing with the interphase. Double the volume of 95% ethanol was added slowly to the aqueous layer and mixed by inverting the tubesfor 3 min to precipitate RNA and centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was slowly decanted and the precipitate was washed by adding 1mL of 70% chilled ethanol and centrifuged at 12,000 g for 15 min at cold condition (4°C).The supernatant was gently decanted and the pellet was air dried and redissolved in a minimum volume of RNase free water and stored at -20°C. Finally, the quality of RNA was checked by formaldehyde gel electrophoresis.

2.4.2.2. CTAB method with LiCl

Hundred mg fresh leaf tissues were surface sterilized with RNase free water for RNA extraction. An eppendorf tube of 1.5 mL with extraction buffer (2% CTAB,2% PVP K30, 100 mM Tris-HCl with pH 8.0, 25 mM EDTA with pH 8.0, 2M NaCl and 05 g/L spermidine) and 300 µL of 2% β-mercaptoethanol was pre-heated at 65C. The tissue was transferred to a sterile pre-chilled mortar and pestle, ground in liquid nitrogen to a fine powder with the heated buffer was added slowly to block the coagulation of the powder. Then, the slurry was transferred to a sterile eppendorf tube and homogenized with high speed for 1 min. An equal volume of chloroform:isoamyl alcohol was added, mixed and centrifuged at 10,000 g for 10 min at room temperature to separate the phase. The upper aqueous layer containing RNA was transferred to a sterile tube

without mixing with the interphase. An equal volume of chloroform: isoamyl alcohol was added and centrifuged in the same way as in theprevious step. To the aqueous supernatant, 0.5 mL of 2M LiCl was added and keptat 5C overnight.

After the overnight incubation, the sample was centrifuged at 10,000 rpm for20 min at 4C. Carefully discarded the supernatant and the tubes were inverted to drain the remaining supernatant onto a clean tissue paper. The pellet was dissolved in preheated SSTE buffer (1M NaCl, 0.5% SDS, 10 Mm Tris-HCl with pH 8.0 and 1mM EDTA with pH 8.0) at 65C. An equal volume of chloroform: isoamyl alcohol was added and mixed thoroughly. The chloroform:isoamyl alcohol was repeated till the precipitate became clear as compared to gel-like nature. To this mixture, double the volume of absolute ethanol was added to precipitate the RNA and incubated at a temperature of -20C for 2-3 h. After the incubation period, the RNA was again centrifuged for 20 min at 20,000 g for 20 min. The supernatant was decantedand the pellet was air dried and dissolved in a minimum volume of RNase free water and stored at -20C. The quality of RNA was checked by formaldehyde gel electrophoresis.

2.4.2.3. RNA isolation by modified GTC method

All the GTC protocol was sequentially carried except the addition of 3% Polyvinylpyrollidone (PVP) in the extraction step. PVP is a high molecular weight polymer that binds to the contaminants such as polyphenolics and polysaccharides that were present in the tissue. The quality of RNA was checked by formaldehyde gel electrophoresis.

2.4.3. cDNA synthesis of teak RNA

One µg of intact RNA isolated by various methods were used to synthesize cDNA to a volume of 25 µL. Initially RNA was denatured in presence of 100 ng of OligodT primer at 65°C for 10 min and snap chilled in ice for 5 min. To the denatured RNA, the following reagents were added: RT buffer- 5.0 µL (1X), dNTPs-2.5 µL (1mM), RNase inhibitor -1.0 µL, mMuLV (20 U/ µL) - 1.0 µL and water - 9.5 µL. The reaction mixture was incubated at 37°C for 1 h and 70°C for 10 min. The c DNA was stored at -80°C till further used.

2.4.4. Semi quantitative RT- PCR analysis (Reverse Transcriptase analysis)

Semi quantitative RT - PCR was attempted to analyze the expression of the anthocyanin genes namely CHS, CHI, F3H, F3′5′H, DFR, ANS, and UF3GT from the young and matured leaf tissues of teak under following PCR cycle: initial DNA denaturation 98°C for 30 sec followed by 30 cycles of amplification (denaturation 98°C for 10 sec; primer annealing 60°C for 30 sec; primer extension 72°C for 30 sec) for 20 µL standard reaction volume. Actin was employed as the positive control for normalization of the amount of templates in PCR cycles. Total 8 µL PCR sample (6 µL PCR products + 2 µL loading dye) was loaded in 2% agarose gel and run at 85V for 90 min.

2.4.5. Quantitative Real Time PCR analysis

RT-qPCR was done using CYBR Green 1 Master and Roche Light Cycler 480 system (Roche Diagnostics India Pvt. Ltd.). Samples of 100 ng of cDNA were employed as template from each leaf sample. The experiment was carried by manufacturer's protocol to prepare 20 µL standard reaction volume. 18S rRNA was employed as indigenous positive calibrator and tomato (Cherry variety) was used as target positive calibrator for normalization of the PCR cycles. Each leaf sample in triplicates was used under the following PCR protocols: 95°C for 10 min followed by 45 cycles of amplification (denaturation 95°C for 10 sec; primer annealing 57°C for 15 sec and primer extension 72°C for 15 sec). Primers employed in the reaction were displayed in the Table 2.

2.5. Statistical analysis

All the data obtained through the study was analyzed by a Three-factor Analysis of Variance set in completely randomized design. At least three replications were maintained for all the treatments in all the cases of experiments. $p < 0.01$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Anthocyanin content

As an initial part of the study, the variation of anthocyanin content from young and mature leaves of teak was quantified according to standard protocol. Anthocyanin content from young leaves of teak is 6.4 mg/g and that of matured leaves is 3.25 mg/g. The pH differential method and HPLC are the stable methods employed for estimating anthocyanin level from plant tissues. Lao and Giusti [7] correlated the efficacy of 4 various methods for the estimation of anthocyanin content. Total anthocyanin level showed wide variations

revealed by the protocols i.e., they documented that extraction medium plays a dominant role in anthocyanin isolation.

Anthocyanin is a unique biomolecule in terms of nutrient, nutraceutical and medicinal attributes. These pigmented molecules show high nutrient value and thereby as drug. As a nutraceutical point of view, its availability in the cells is a critical factor for retaining the ideal health value and blocking age related disorders. Murukan and Murugan [8] substantiated the antioxidant power of teak anthocyanin using different *in vitro* antioxidant scavenging protocols. Anthocyanins are antimetastatic molecules and also proven anti-angiogenesis factors as revealed by the *in vitro* cell culture and rat/mice model studies. Antiangiogenesis controls the production of new blood vessels that channelize O_2 to the onco cells [9, 10].

3.2. Quantification of phenyl alanine ammonialyase (PAL), chalconeisomerase (CHI) and UDP-glycosyltransferase (UGT) in the young and matured teak leaves

The key enzyme of the anthocyanin synthetic pathway is phenyl alanine ammonia lyase (PAL). Remarkable PAL activity was noted on the young leaves i.e., 3267.87 U/g than the matured leaves (833.65 U/g) (Table 3). Similarly, CHI the regulatory enzyme displayed an activity of 642.7 U/g in the young leaves when compared to the matured leaves 118.4 U/g (Table 3). In addition, UGT the endpoint enzyme that catalyzes glucosyl transfer from UDP-glucose to 3-hydroxylgroup to produce the stable cyanine glucosides. It was remarkably increased in the young leaves (534.54 U/g) than matured leaves (116.74 U/g). The entire data were statistically significant ($p < 0.01$, Table 3). The F values were 2388.62** (PAL), 854.87** (CHI) and 789.68** (UGT) respectively. The corresponding CD valueswere 1.0472, 0.88246 and 1.132.

Table 3: Enzyme activity profile of PAL, CHI and UGT of the young and matured teak leaves related with anthocyanin synthesis

	PAL (U/g)	CHI (U/g)	UGT (U/g)
Young	3267.87	642.7	534.54
Matured	833.65	118.4	116.74
F ratio	2388.62**	854.87**	789.68**
CD	1.0472	0.88246	1.132
SE	0.8944	0.3247	0.7083

***The values were statistically significant at $p < 0.01$*

3.3. Expression Analysis of Anthocyanin Biosynthetic Genes

3.3.1. RNA isolation from young and matured leaves

3.3.1.1. Isolation of RNA by GTC method

RNA was isolated from the teak leaves, as per the protocol of GTC. The supernatant prior to the centrifugation was appeared reddish with more intensity in the tender leaves, indicating the interference of phenols and tannins. In the formaldehyde agarose gel the RNA was found degraded without 18S and 28S rRNA discrete bands confirming the poor quality of RNA (Fig 1a).

3.3.1.2. Isolation of RNA by LiCl method

Due to the degradation of RNA by GTC method, LiCL with CTAB was tried for checking the quality of RNA. Due to the lengthy procedure, special care was taken at each step for maintaining the quality of RNA. At the last step prior to chloroform: isoamyl alcohol washing, a coloured thick jelly precipitate appeared during isolation. The RNA was isolated from the jelly by repeated washing with the solvent and was documented in the agarose gel. Figure 1b displays the nature of RNA isolated from tender and matured leaves of teak by LiCl method. Though the gel picture of RNA showed little more resolution than the RNA of GTC method, the18S and 28S rRNA bands are not discrete, suggesting the quality of RNA was not sufficient for further screening.

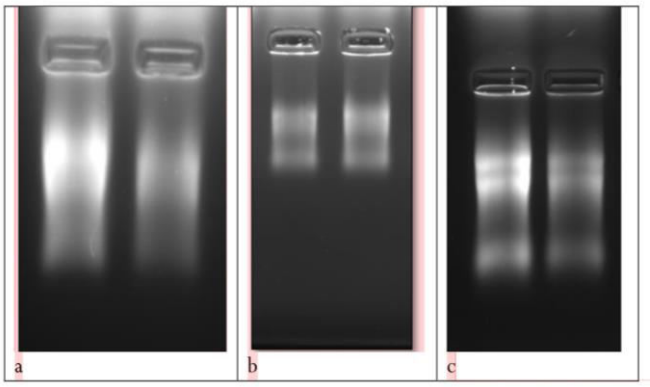
3.3.1.3. Isolation of RNA by modified GTC method

RNA isolation was extended using a modified GTC procedure using PVP i.e., addition of 3% PVP in the extraction buffer along with GTC has showed significant improvement in the isolation procedure. The colour of the final supernatant and jelly nature were disappeared and clear RNA pellet was obtained. The agarose gel electrophoresis of RNA pellet showed two clear discrete bands of 18S and 28S rRNA confirming the good nature of RNA (Fig. 1c). Thus, the standardized modified protocol of GTC using PVP yielded RNA from teak leaves was employed further for molecular analysis.

3.3.2. Expression analysis of anthocyanin biosynthetic genes

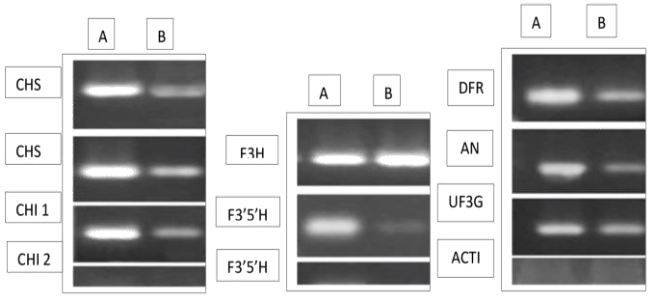
Fresh young and matured leaves of teak were chosen for expression analysis of anthocyanin biosynthetic genes. Leaves are unique in terms of the presence of chloroplast. Many genetical changes occur in the leaves of teak in relation to their colour (young to matured

stages) and there by the expression of genes [11]. Differential gene expressions were noticed in the two stages of leaves as reflected in terms of intensity of bands and also from the data of semi-quantitative PCR results (Fig. 2). Expression of both *CHS*1 and *CHS*2 were higher in the young leaves as compared to the matured stage (low expression of *CHS*1 and *CHS*2). Similarly, *CHI1* and *CHI2* were also expressed soundly in the young leaves as compared to matured leaves. Meanwhile, the expression of *F3H* gene was remarkable both in the young and matured leaves. In contrast, *UF3GT* showed more expression in the young leaves than matured leaves. Level of expression of *F3′5′H1*, *F3′5′H2*, *DFR*and *ANS* were also found significant in the young leaves than the matured leaves.



(a) GTC method, (b) LiCl method and (c) modified GTC method (Lane 1 - young leaf, Lane 2 - matured leaf)

Fig. 1: RNA isolated from young and matured leaves of teak



A-Young leaf, B - Matured leaf, Actin was used as control

Fig. 2: Semi-quantitative RT-PCR analysis of anthocyanin pathway genes from teak

Relative gene expression was also analyzed through RTqPCR in terms of variations in the fold of expression (Fig. 3). Amount of a gene expression levels (in terms of folds) was higher in the young leaves as compared to matured. Expression of both *CHS*1 and *CHS*2 was higher in the young leaves than matured leaves (6 and 5 folds higher respectively). Expression levels of both *CHI*1 and *CHI*2 showed a decrease towards maturity of leaves. Further, with in the copies of young leaves *CHI*2 showed up to 4.5 fold higher expression than *CHI*1. The expression of *F3H* was more or less similar in the young and matured leaves.The expression of *F3′5′H1* was 5 fold higher in the young leaves thanmatured leaves. In contrast, *F3′5′H2* showed up to eleven fold higher expression in the young leaves. *DFR* and *ANS* genes are immediate precursors of anthocyanin that transforms dihydromyricetin to leucodelphinidin and leucodelphinidin to delphinidin respectively [12].

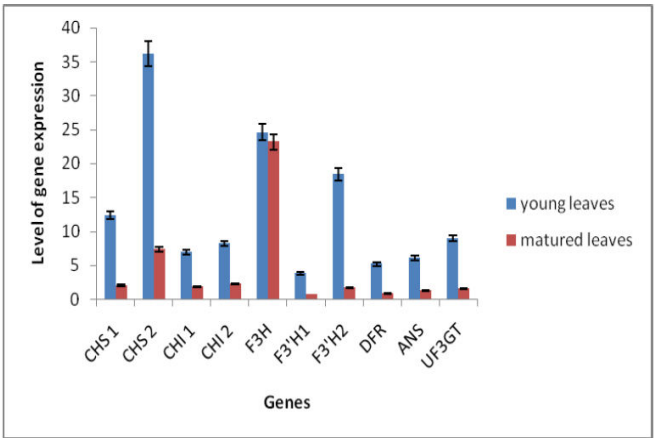


Fig. 3: qRT-PCR analysis of level of gene expression from young and matured leaves of teak

In the present study, *DFR* showed an upregulation up to six fold and *ANS* showed up to five fold increased expression in the young leaves. *UF3GT* showed up to 6 fold increased level of expression in the young leaves. 18S rRNA was used in this study as internal control for RT-qPCR analysis due to its relatively high expression and low variations of expression as compared to other housekeeping genes [13]. Cherry tomato was employed as positive control and also the fruits showed different maturity stages. Further, the species displayed many changes during their cultivation across the regions. Like teak anthocyanin pathway, genes of tomato were also documented. Contrarily, the quantity of anthocyanin accumulation was low and was due to the poor expression of *CHI*. Interestingly, the tomato produce delphinidin types of anthocyanin in their leaves but not in the fruits. Thus, the expression of *CHI*in the fruits might limit the anthocyanin production in the cultivated

tomato [14]. In the present study, increased expression of *CHI*1 and *CHI*2 in the young leaves was noticed. Interestingly, *CHI*1 showed two fold lower expressions than *CHI*2 in the matured leaves.

4. CONCLUSION

As an initial part of the study, anthocyanin content was quantified from different parts of the teak plants. Interestingly, the young leaves contain remarkable anthocyanin content as compared to others.The major anthocyanin pathway key enzymes such as phenyl alanineammonialyase (PAL), chalconeisomerase (CHI) and UDP-glycosyltransferase (UGT) were assayed. Remarkable activities were noticed with young leaves as compared to matured. The data further substantiating anthocyanin content in the young leaves. The Rt- PCR results of the chalcone synthase, chalconeisomerase, flavanone 3-hydroxylase, dihydrofolatereductase, anthocyanindin synthase and UDPG-flavonoid 3-glucosyl transferasegenes up regulation corroborates with semi quantitative PCR data. Higher expression of chalcone isomerase in the young leaves might be the reason of the conversion of chalcone into naringenin which in turn into the production of anthocyanin. RTqPCR indicated optimal expression of dihydrofolate reductase and anthocyanindinsynthase in the young leaves than the matured substantiates the sufficient substrate conversion in the anthocyanin pathway. From the results obtained, the study concludes that majority of the genes involved in anthocyanin biosynthetic pathway showed high expression profile in young leaves than matured ones. The study can be extended for the sequencing of major anthocyanin upregulated genes such as CHS, DFR, ANS and CHI and thereby open a way to the increased production of anthocyanin commercially.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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