Research Article

Bao-Jun Zhu, Qian Wang, Jing-Hui Wang, Lin-Lin Gao, Jing-Wen Zhang and Ru-Qiang Huang

DFR and PAL gene transcription and their correlation with anthocyanin accumulation in Rhodomyrtus tomentosa (Aiton.) Hassk.

Abstract

Objectives: Rhodomyrtus tomentosa (Aiton.) Hassk. (R. tomentosa) is rich in nutrients and has multiple pharmacological applications. Anthocyanins confer color to the flowers and berries of R. tomentosa and provide protection against photodamage. The dihydroflavonol 4-reductase gene (DFR) and phenylalanine ammonia lyase gene (PAL) are crucial for anthocyanin synthesis.

Methods: DFR and PAL transcript levels and anthocyanin content in the pigmented organs of R. tomentosa were investigated through qRT-PCR analysis and spectrophotometry, respectively. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was selected as the reference gene for the normalization of DFR and PAL transcript levels.

Results: Transcript levels of DFR and PAL were higher in organs with vigorous metabolism than those in senescent organs. DFR and PAL transcript levels were up-regulated during the initial and middle-maturity periods of fruit. These expression patterns are consistent with fruit color development. The highest transcript levels of PAL and DFR were observed during the middle-maturity period or the red-fruit period.

Conclusion: During the late maturity period of R. tomentosa fruit, the transcript levels of the two genes were down-regulated even though anthocyanins were continuously accumulated, which was different from the accumulation of anthocyanins in some late mature fruits.

Keywords: Rhodomyrtus tomentosa (Aiton.) Hassk; Anthocyanins; Phenylalanine ammonia lyase gene; Dihydroflavonol 4-reductase gene; Reference gene; qRT-PCR.

References

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Amaç: Rhodomyrtus tomentosa (Aiton.) Hassk. (R. tomentosa) besin açısından zengindir ve çoklu farmakolojik uygulamaları sahiptir. Anthosiyainler, R. tomentosa’nın çiçek ve meyvelerine renk katmakta ve ışık hasanına karşı koruma sağlamaktadır. Dihidroflavonol 4-redüktaz geni (DFR) ve fenilalanin amonialyaz geni (PAL) antosiyainin sentezi için çok önemlidir.

Gerek ve Yöntem: R. Tomentosa’nın pigmentli organlarında DFR ve PAL transkript seviyeleri ve antosiyainin içeriği sarsıyla qRT-PCR analizi ve spektrofotometri ile incelendi. Gliseraldehit-3-fosfat dehidrogenaz (GAPDH) geni, DFR ve PAL transkript seviyelerinin normalleştirilmesi için referans gen olarak seçilmiş.

Sonuç: R. tomentosa meyvesinin geç olgunlaşma dönem- side, iki genin transkript seviyeleri, antosyaninlerin sürekli olarak birikmesine rağmen, bazı geç olgun mey- velerdeki antosyanin birikiminden farklı olarak, aşağı- düzelenmiştir.

Anahtar Kelimeler: Rhodomyrtus tomentosa (Aiton.) Hassk; Antosyaninler; Fenilalanin ammonialyaz geni; Dihidroflavonol 4-redüktaz geni; Referans gen; qRT-PCR

Introduction

Medicinal and aromatic plants is one of the important and paying branches of agriculture and has been practiced in most of the countries in the world since centuries. Medicinal and aromatic plants have been used not only for nutrition purposes but also to meet personal and social needs such as curing diseases, etc. The medicinal and aromatic plants a lots of phytochemicals that important for human health [1–3]. Rhodomyrtus tomentosa (Aiton) Hassk., a traditional evergreen shrub with rose-pink flowers and dark-purple bell-shaped fruit, is commonly distributed in Southeast Asia [4]. Rhodomyrtus tomentosa is used in folk medicine for colic diarrhea, dysentery, abscesses, traumatic hemorrhage, gynaecopathies, and immune disorders [5, 6]. The majority of functional components, including volatile oil, polyphenols, and polysaccha- rides of R. tomentosa have been isolated and character- ized. Although anthocyanins are the more abundant and important polyphenols in R. tomentosa, the molecular mechanism of anthocyanin biosynthesis in this plant has not been described. Molecular research has unveiled an unexpected wealth of differentially expressed and regu- lated genes in anthocyanin biosynthesis and accumula- tion in R. tomentosa.

Rhodomyrtus tomentosa berries contain various poly- phenols, including anthocyanins, flavonols, ellagitanin, gallic acid, and piceatannol [7–9]. Anthocyanins from R. tomentosa mainly consist of delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, cyanidin 3-O-pentoside, pelargonin 3-O-glucoside, petunidin 3-O-glucoside, peo- nidin 3-O-glucoside, and malvidin 3-O-glucoside [4]. Anthocyanins not only regulate red–blue color pigmen- tation in many plant tissues but also have an important role in seed dispersion, UV protection, and antioxidation [10]. However, the molecular regulators that control anthocyanin accumulation in R. tomentosa berries remain undefined.

The anthocyanin biosynthetic pathway is highly conserved among different plant species and is a branch of the phenylpropanoid pathway. PAL is the first key enzyme gene and the rate-limiting enzyme gene in anthocyanin synthesis and accumulation. Moreover, it contributes to stress resistance and the maintenance of normal physiological functions. PAL is mainly expressed in the roots and in the blooming floral organs, with little expression in the aged leaves. PAL transcript level is related to anthocyanin accumulation in colored organs, and PAL expression is up-regulated during the maturation of Malus and Allium cepa fruits [11, 12].

DFR is an important downstream gene of the antho- cyanin biosynthetic pathway and determines the type and structure of anthocyanin to be generated. DFR possesses an NADPH binding site (VTGADFIGSWLIMRLLERGY) and substrate binding site (TVKRLVFTSSAGTLNVQPQQK) [13]. The substrate binding site specifically recognizes dihydrokaempferol, dihydroquercetin, or dihydromyricetin. It catalyzes different substrates into lecoanthocyani- din and accounts for the inability of Petunia hybrida and Cymbidium hybrida to generate pelargonidin [13].

PAL and DFR have been isolated and characterized from numerous plants, including Arabidopsis thaliana, P. hybrida, and Zea mays [14–16]. Many members of the PAL and DFR superfamilies contribute to anthocyanin syn- thesis and accumulation, and some members participate in plant protection strategies against external environmental stress. However, the molecular event that under- lies anthocyanin accumulation in R. tomentosa remains unclear. In this study, we isolated and characterized PAL and DFR. We investigated the transcript levels of these genes in the pigmented organs of R. tomentosa and identified their correlations with anthocyanin accumulation in R. tomentosa.

A suitable reference gene for the normalization of gene transcript levels is necessary for accurate and reli- able data analysis [17, 18]. A housekeeping gene with known properties, participation in maintaining basic cellular function, and ubiquitous expression in all cells of an organism is usually used as a reference gene [17, 19, 20]. Glyceraldehyde-3-phosphate (GAPDH), ribosomal RNA (18S rRNA), actin (ACT), elongation factor 1-α, poly- ubiquitin, and tubulin (TUB) are typical and classical reference genes that have been isolated and identified.
from many plants [21, 22]. However, a gene with constant and uniform transcript level does not exist. Almost all genes have condition-specific, tissue-specific, developmental-specific, or cultivar-dependent transcriptional patterns [21]. For example, *Actin* and *18S rRNA* are constantly transcribed in *Cichorium intybus* L. but not in *Prunus persica* L. Batsch [23]; *TUB* is stably transcribed in *Cucumis sativus* L.; its transcription, however, is destabilized by hormone and stress treatments [24]. *18S rRNA* and *25S rRNA* are stably transcribed during seedling growth but not throughout the whole growth phase [25].

We have previously successfully isolated and characterized volatile oils and flavonols from *R. tomentosa* [8, 26]. In the present study, we aimed to quantify the transcript levels of structural genes in the phenylpropanoid pathway and illustrate the relationships between these genes and anthocyanin accumulation. We cloned *18S rRNA*, *Actin*, *GAPDH*, *PAL*, and *DFR*; selected a suitable reference gene for normalization from *18S rRNA*, *Actin*, and *GAPDH*; and studied the transcript levels of *PAL* and *DFR* genes and their relationships with anthocyanin accumulation.

**Materials and methods**

**Reagents**

Kits and main reagents included Plant Total RNA extraction kit (OMEGA company), as well as SYBR® Premix Ex Taq II, Taq™ Hot Start Version, Premix Taq™ (LA Taq™ Version 2.0 plus dye), PrimeScript™ II 1st Strand cDNA Synthesis Kit, and PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect for Real Time, TAKARA company). Software for bioinformatics analyses included BLAST (online tool), ORF Finder (online tool), DNAStar, CLustal X 2.1, MEGA 6.0, GeNorm, NormFinder, Bestkeeper, GeneDoc, and ABI 7500 V2.0.

**Plant materials**


Tender leaves (TL), growing leaves (GL), flourishing petals (FP), late-flowering petals (LFP), green fruits (GF), red fruits (RF), and purple fruits (PF) of *R. tomentosa* were acquired from Jilong Hill, which is located in Guangdong Academy of Agricultural Sciences. The plant was validated as *R. tomentosa* (Aiton.) Hassk (family Myrtaceae, Rhodomyrtus) by Guangdong Academy of Agricultural Sciences. Materials were first cooled in liquid nitrogen and then preserved in a −80°C low-temperature freezer. The degradation of total RNA was prevented with the addition of RNase inhibitors.

**Relative anthocyanin content**

Relative anthocyanin content was measured using the pH differential spectrophotometric method by Weiss with minor modifications [27]. Accurately weighed 50 mg eight kinds of fruits samples were soaked in 1 mL 1% hydrochloric acid in methanol (v/v), and extracted at 4°C for up to 16 h. Samples were then centrifuged at 15285 g for 8 min. The absorbance was measured in a Microplate reader at 530 nm and at 657 nm. The relative anthocyanin content of the diluted sample was then calculated as follows:

\[
\text{Relative anthocyanin content (ΔA·g}^{-1}) = \left[\text{OD}_{530} - (0.25 \times \text{OD}_{657})\right] \times 20 \times \text{dilution multiple} \tag{1}
\]

**Primer design**

Genes used in this research were obtained through reviews involving plant *18S rRNA*, *ACT*, *GAPDH*, *PAL*, and *DFR*, or related genes retrieved from the National Center of Biotechnology Information database (NCBI). Subsequent alignments were analyzed using DNAStar and GeneDoc software. Primers were designed using Prime Premier 5.0 software and Primer-Blast online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) with the following parameters: (a) product size range of 80–300 bp, (b) primer size of 18–25 bp, and Tm value in the range of 58–62°C [28]. The primer’s specificity and expected amplicon size were validated through electrophoresis on 1.5% agarose gel stained with GelRed. DNA sequences were confirmed through sequencing. Primers used in this study are as follows:
Total RNA extraction and gene cloning

A mortar and pestle were used to ground TL, GL, FP, LFP, GF, RF, and PF samples in liquid nitrogen. Powders were fragmentized using beta-mercaptoethanol to form LFP, GF, RF, and PF samples in liquid nitrogen. Powders A mortar and pestle were used to ground TL, GL, FP, LFP, GF, RF, and PF in accordance with the instructions included with Plant RNA Kit (OMEGA Company). Total RNA extraction were performed in accordance with the instructions included with Plant RNA Kit (OMEGA Company). Total RNA quality was checked using 1.5% agarose stained with GelRed. The integrity of total RNA was confirmed through UV–VB spectrophotometry. The integrity of gene products were confirmed through electrophoresis on 1.5% agarose gel stained with GelRed. PCR products of Rh 18S rRNA, Rh Actin, Rh GAPDH, and Rh PAL were sent out for sequencing. Given that primers used for DFR amplification are degenerate primers, DFR PCR products were first recovered and purified using an agarose gel DNA extraction kit. After purification, the PCR product was ligated to pMD18-T vector for the construction of a recombinant plasmid. The recombinant plasmid was then transduced to competent Escherichia coli DH5α. The reaction mixture, which contained recombinant E. coli, was plated on LB culture medium with X-Gal, IPTG, and ampicillin for blue–white selection. Ten white colonies were selected for PCR amplification after 16 h of culture at 37°C. After confirming PCR products on 1.5% agarose gel stained with GelRed, bacterial fluids were sent for sequencing. Bioinformatics analyses for cloned genes were conducted using NCBI-BLAST. Phylogenetic trees were constructed using MEGA6.0.

Reference gene selection

Total RNA was extracted from TL, GL, FP, LFP, GF, RF, and PF in accordance with the above RNA extraction method. Genomic DNA (gDNA) was removed from total RNA in accordance with the instructions included with the RT reagent kit. First, 900 ng total RNA was added to 2.0 μL 5-fold gDNA Eraser Buffer, 1.0 μL gDNA Eraser, and RNase-free dH₂O to prepare a 10 μL solution for gDNA removal. First-strand cDNA was synthesized after further gDNA removal. All procedures were conducted under frozen conditions. First, 20.0 μL reaction solution was prepared with 10.0 μL of the above gDNA removal solution, 1.0 μL PrimeScript RT Enzyme Mix I, 1.0 μL RT Primer Mix, 4.0 μL 5×PrimeScript Buffer 2 (for Real Time), and 4.0 μL RNase-free dH₂O. The reaction solution was then reacted for 15 min at 37°C then at 85°C for 5 s in a thermal cycler.

18S rRNA, ACT, and GAPDH transcript levels in R. tomentosa TL, GL, FP, LFP, GF, RF, and PF were investigated through qRT-PCR in 96-well plates with ABI 7500 Real-Time PCR system using SYBR® Premix Ex Taq II (TAKARA). The 20.0 μL reaction volume contained 10.0 μL SYBR® Premix Ex Taq II (Tli RNaseH Plus), 1.6 μL primers.
(10 μM), 2.0 μL appropriately diluted cDNA template, and 6 μL ddH2O. Cycling conditions were as recommended by the apparatus: 95°C 30 s for initial denaturation and 5 s at 95°C followed by 34 s at 60°C for 40 cycles. At the end of the run, melting curves were generated by heating the amplification products from 60°C to 95°C. All samples were run in technical duplicates and biological triplicates. The specificity of all qRT-PCR primers was validated by the amplification of melting curves. The expression levels of each gene was confirmed as the cycle number needed for the fluorescent signal to reach a threshold fixed in the exponential phase of the PCR reaction (Ct value) [29]. Data of each gene were analyzed using GeNorm [30], NormFinder [17], and BestKeeper software. The most stably expressed gene out of the three candidates was selected as the reference gene for the normalization of target gene expression in R. tomentosa.

Transcriptional characterization of PAL and DFR

FP, LFP, RF, and PF samples of R. tomentosa of the relative content of total anthocyanin was calculated using the following equation 1. PAL and DFR transcript levels in TL, GL, FP, GF, RF, and PF were investigated. RNA extraction, cDNA synthesis for real-time PCR analysis, and transcript level analysis were performed in accordance with the above methods. qRT-DFR and qRT-PAL primers were used for amplification. Data on relative gene transcript levels were analyzed through 2−ΔΔCt method. All samples were run in technical duplicates and biological triplicates.

Results and discussion

Anthocyanin content comparison

In this study, eight kinds of fruits were studied: Vaccinium vitis-idaea Linn, Prunus salicina Lindl, Rhodomyrtus tomentosa (Aiton) Hassk, Vitis romanetii Rom. Caill, P. Prunus pseudocerasus, Crataegus pinnatifida, Red delicious apple, Amygdalus persica (L.) Batsch. All fruits were mature and stored at −20°C (no more than 1 month) until extraction and analysis. It is known from Figure 1, Vaccinium vitis-idaea Linn had the highest anthocyanin content in the fruits tested, followed by Prunus salicina Lindl and R. tomentosa.
GAPDH was approximately 400 bp in size; and that of DFR was approximately 500 bp in size. The observed product sizes are consistent with the expected product sizes.

The DFR PCR product was cloned into the pMD-18T vector to form recombinant plasmids. Recombinant plasmids were then transduced to competent E. coli DH5α to construct recombinant E. coli DH5α for sequencing.

Bioinformatics analyses of cloned genes were performed using Nucleotide BLAST and MEGA 6.0. The PAL sequence of R. tomentosa exhibited 95% similarity with that of Syzygium malaccense (Accession: GU233756.1), 94% with that of E. grandis (Accession: XM_010033665.1), 86% with that of V. vinifera (Accession: EF192469.1), and 84% with that of Prunus armeniaca ‘Harcot’ (Accession: EF031063.1). The DFR sequence of R. tomentosa exhibited 92% similarity with that of L. grandis (Accession: XM_010033665.1), 91% with that of L. exilicaulis (Accession: AJX944786.1). The N-J phylogenetic tree of PAL gene and DFR gene (Figure 3A and B) was constructed by MEGA 6.0 with 1000 bootstrap replicates. R. tomentosa genes and corresponding Myrtaceae genes grouped into a clade that included R. tomentosa, E. grandis, S. malaccense, S. maiire, and other plant species in Myrtaceae. After basic bioinformatics analysis, cloned genes were submitted to NCBI for indexing. The accession numbers of PAL and DFR fragments are KU298502 and KU233523 respectively.

Reference gene selection

18S rRNA, Actin, and GAPDH were considered as candidate genes for reference gene selection. The transcript levels of these genes in different R. tomentosa organs were investigated through qRT-PCR. The specificity of all qRT-PCR primers was validated through 1.5% agarose gel electrophoresis and melting curve amplification. Results indicated that the melting temperature of all primers ranged from 80°C to 85°C, and each melting curve possessed a single peak.

The transcriptional stability of the three candidate genes were evaluated using geNorm, NormFinder, and BestKeeper. Data were first analyzed by geNorm. Average expression stability level (M) was calculated in this analysis. Genes with the lowest stability of transcriptional levels (the highest M value) out of all candidate genes were excluded until two most stable expressed genes remained. Table 1 shows the M values of the remaining control genes. Results indicated that 18S rRNA is the least stable gene out of the three candidate genes and had an M value of 294.

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0.297. By contrast, GAPDH and Actin genes are relatively stable genes with \( M \) values of 0.193 and 0.192, respectively. All stable values in this study are below the default limit \( M = 1.5 \) [33], illustrating that the results are highly reliable. Furthermore, pairwise variations (\( V_{2/3} \)) were calculated in geNorm to determine the optimal numbers of reference genes for normalization. The \( V_{2/3} \) value was 0.098, which is below the default limit of 0.15. This result indicated that a combination of two reference genes is suitable for normalizing gene expression in \( R. \) tomentosa. Thus, additional reference genes are not required for normalization under normal experimental conditions (Table 1). Using NormFinder, the stability of candidate genes was ranked in accordance with the variation in their expression levels. Out of the three candidate genes, 18S rRNA is the least stable gene with the highest stability value of 0.164, whereas GAPDH is the most stable gene with a stability value of 0.059 (Table 1).

The BestKeeper program index was generated using geometric mean of candidate gene (\( C_r \)) values. \( C_r \) values were used to calculate the variation in gene expression and standard deviation (SD value). Genes with SD values greater than 1.0 were considered as transcription [33]. BestKeeper highlighted Actin as a potential reference gene with a stability value of 0.83, then GAPDH with a stability value of 0.84. 18S rRNA had the highest \( C_r \) value variation with a stability value of 1.415. These results indicated that actin is a suitable reference gene. Thus, given its comprehensive rank of top one, GAPDH was selected as a suitable reference gene under experimental conditions.

### Characterization of PAL and DFR transcript levels and their correlation with anthocyanin accumulation

Transcript levels of PAL and DFR genes in different organs of \( R. \) tomentosa were investigated through qRT-PCR analysis with GAPDH as a reference gene. Results (Figure 4) indicated that transcripts of both genes are present in the tested leaves, flowers, and fruits. However, the transcript levels of these genes are tissue-specific. The highest DFR transcript level was observed in RF, followed by those in RF, TL, PF, GF, and GL. However, almost DFR transcripts were detected in LFP. By contrast, PAL transcript levels were highest in RF, followed those in by TL, PF, FP, GF, and GL. Low PAL transcript levels were found in LFP.

Results showed high transcript levels of PAL and DFR in organs with vigorous metabolism but not in senescent

<table>
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<th>Gene name</th>
<th>Stability value (geNorm)</th>
<th>Ranking order (geNorm)</th>
<th>Stability value (NormFinder)</th>
<th>Ranking order (NormFinder)</th>
<th>Stability value (BestKeeper)</th>
<th>Ranking order (BestKeeper)</th>
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<td>2</td>
<td>0.83</td>
<td>1</td>
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<tr>
<td>GAPDH</td>
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<td>0.059</td>
<td>1</td>
<td>0.84</td>
<td>2</td>
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![Figure 4: Tissue-specific expression of DFR and PAL genes in R. tomentosa.](image)

TL, tender leaves; GL, growing leaves; FP, flourishing petals; LBP, late-flowering petals; GF, green fruits; RF, red fruits; PF, purple fruits.
organs. Transcript levels of DFR and PAL in floral organs indicated that gene transcription is higher during the flourishing period than that during the late flowering period. The transcription patterns of the two genes are consistent with the floral pigmentation process and anthocyanin accumulation. During the fruit maturity period, DFR and PAL transcript levels were first up-regulated and then down-regulated until peaking during the middle period (red-fruit period). During the initial and the middle periods of fruit development, the transcript levels of the two genes were up-regulated, and the red coloration of the fruit intensified. Results indicated that the transcript levels of these two structural genes are consistent with anthocyanin accumulation. DFR and PAL transcript levels were down-regulated during the late maturity period. Anthocyanins, however, continuously accumulated in fruits. These results indicated that the transcript levels of the target genes and anthocyanin accumulation during the late maturity period are not correlated. We inferred that both genes are highly transcribed during the initial (green-fruit period) and the middle (red-fruit period) maturity periods for synthase synthesis. During the late maturity period, DFR and PAL syntheses catalyze substrates to synthesize anthocyanins. Therefore, the transcript levels of genes contribute directly to anthocyanin accumulation during the initial and middle maturity periods. DFR and PAL syntheses promote anthocyanin accumulation in fruit during the late maturity period. Hence, anthocyanin content continually increased even though the expression levels of DFR and PAL genes decreased.

Anthocyanidin is a water-soluble pigment that is widely present in the plant's coloring organs. As the pH of the cell liquid changes, the plant's anthocyanin appears red, blue, or purple, which makes the flowers and fruits colorful and colorful. The relative contents of total anthocyanins in different fruits were very different, and the content of anthocyanins in blue, purple and red fruits was higher than that in yellow and orange fruits. The anthocyanins content of R. tomentosa is higher in 8 kinds of fruits tested. When the R. tomentosa fruit ripened late, the expression levels of DFR and PAL genes decreased, and the anthocyanin content increased continuously. However, grape anthocyanins concentration may decrease slightly during over-maturing [35]. The anthocyanin concentration from the grape also increased during the ripening step until reachinga maximum value, and then it began to diminish [36]. As apricots ripened, anthocyanin concentrations increased, reached a maximum and decreased toward the end of maturation phase [37]. This is consistent with the findings reported for lychee pericarp [38].

PAL is the first and rate-limiting gene in the anthocyanin biosynthetic pathway and is also involved in stress resistance and anti-retroviral responses [39, 40]. DFR transcript levels determine the type of anthocyanin that is synthesized and anthocyanin accumulation. DFR and PAL are highly expressed in flourishing petals with rose-pink color but are poorly expressed in late-flowering petals with light pink or white color. This result indicated that the transcription pattern of DFR and PAL are consistent with the pigmentation process of R. tomentosa floral organs. The results of the present research are consistent with those of a previous study [41]. DFR and PAL transcripts are present in fruits and are gradually up-regulated from the initial maturity period (green-fruit period) to the middle maturity period (red-fruit period). This transcriptional pattern is consistent with anthocyanin accumulation. However, the transcript levels of the two genes were down-regulated from the middle maturity period to the late maturity period (purple-fruit period). This pattern opposed anthocyanin accumulation. A previous study obtained similar results for peach coloration and found that the transcript levels of genes involved in anthocyanin biosynthesis are up-regulated during the initial and the middle maturity periods to enable the synthesis of corresponding proteins or synthetases; during the late maturity period, existing synthetases continue to promote anthocyanin biosynthesis and accumulation even though PAL and DFR transcript levels have been down-regulated [42]. In conclusion, we characterized the transcriptional pattern of two structural genes in the anthocyanin biosynthetic pathway of R. tomentosa. Our results enhance the depth of current knowledge on anthocyanin biosynthesis in R. tomentosa and advance research on this medicinal plant.

Conclusions

Rhodomyrtus tomentosa, a medicinal plant with abundant anthocyanin content, is ubiquitous in South China. Molecular investigation revealed that the GAPDH gene is the most stable gene among three candidate genes and thus can be used as a reference gene for the normalization of DFR and PAL expression levels. DFR and PAL transcription is tissue-specific. The transcriptional patterns of DFR and PAL are consistent with the development of pigmentation in leaves, flowers, and fruits at the early maturity stage but not with that in fruits at the late maturity stage. During the late ripening period of P. tomentosa fruit, the transcriptional levels of PAL and DFR were down-regulated, while the anthocyanin accumulation continued,
which was different from the accumulation of anthocyanins in some late ripening fruits.

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**Conflict of interest statement:** The authors declare that there is no conflict of interests regarding the publication of this paper.

**References**


