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Determination of Expression Level of Genes Associated with Anthocyanin Biosynthesis in Some Myrtle (*Myrtus communis* L) Genotypes[#]

Emine Açar[1,a,*], Yıldız Aka Kaçar[2,b]

[1]*Department of Biotechnology, Institute of Natural and Applied Science, Çukurova University, 01330 Adana, Türkiye*
[2]*Department of Horticulture, Faculty of Agriculture, Cukurova University, 01330 Adana, Türkiye*
[*]*Corresponding author*

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ABSTRACT

The Myrtle plant (*Myrtus communis* L.) is a plant species of the Myrtaceae family and a member of the maquis community, which naturally spreads in Mediterranean regions. Being resistant to arid conditions, the ecological distribution areas of the myrtle plant have been allowed to expand. The myrtle plant has been used of medicinal and aromatic plants, having interesting and beautiful flowers, and rich nutrient content of the fruit in terms of valuable phytochemicals, in particular, the nutritional content of its fruits and valuable metabolites have allowed the myrtle plant to be among the healthier foods. Antioxidant activity, which neutralizes reactive oxygen species (ROS), which causes many medical problems, is one of the most important features of the myrtle plant. Investigation of the biosynthesis of anthocyanin, which leads to antioxidant activity, and determination of the biosynthesis in different tissues and genotypes is important, especially in the development of production activities. Furthermore, this study aimed to investigate the anthocyanin biosynthesis in different genotypes with white and black fruits and various tissues of genotypes. For this purpose, the expression levels of *CHS*, *CHI*, *F3H* and *PAL* genes, which are involved in the anthocyanin biosynthesis pathway, were determined by qRT-PCR. In the study, it was determined that there was an increase in the level of genes related to the biosynthesis of anthocyanin in the leaf and fruit tissues of the genotype with white fruits. It was determined that the expression level of genes related to the biosynthesis of anthocyanin was observed to be higher in the leaf and fruit tissues of the genotype with black fruits, and the highest gene expression level was found in black fruits. It was observed that anthocyanin biosynthesis was synthesized in different tissues of the plant, and anthocyanin biosynthesis was higher in fruits compared to leaves.

[a] *acaremine01@gmail.com* *http://orcid.org/0000-0001-9810-9626* | [b] *yildizakakacar01@gmail.com* *http://orcid.org/0000-0001-5314-7952*

Introduction

Technological developments and adverse ecological conditions have led to extraordinary changes in human life. The most important changes are seen in human nutrition. The increase in the world population and the decrease in the areas allocated to agricultural lands restrict access to food. The risk of insufficient food and changing living conditions with technological developments lead people to healthy foods, so the demands for healthy foods are increasing day by day. Fruits and vegetables are among the healthiest foods. Fruits and vegetables contain vitamins, minerals and valuable phytochemicals (Sezgin, 2014). Phytochemicals are important nutritional components and indispensable health elements. Flavonoids constitute the largest class of valuable phytochemicals found in fruits and vegetables. Flavonoids constitute a subclass of phenolic compounds and contain many different groups. Flavones, flavols, isoflavones and anthocyanins are some members of this group (Janićijević et al., 2007; Medda et al., 2021). Anthocyanins are water-soluble pigments that cause plant tissues to take on different colors from pink to purple. Anthocyanidins that make up the anthocyanins that allow plant tissues to be colored on a wide scale are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. Although there are 6 different anthocyanidins, more than 540 anthocyanin-derived pigments have been identified in nature. The basis of these differences depends on the hydroxyl groups in the structure and the sugar and organic acid units attached to these groups. In the phenolic compounds found in the aromatic rings of anthocyanins, the blueness increases as the –OH group increases, and the redness increases as the -OCH3 increases (Fulcrand et al., 1996; Wrolstad et al., 2005) The colors of herbal tissues and the quality of these colors lead to increases in the market prices of herbal products and thus to an increase in economic inputs. For this reason, researches on anthocyanin biosynthesis are intensively carried out. In the

past years, enzymatic studies have been carried out to understand anthocyanin biosynthesis.

Nowadays, researches on the anthocyanin biosynthesis pathway and the genes involved in the biosynthesis pathway, determining the expression level of these genes, understanding the gene expression level and the amount of anthocyanin in plant tissues are gaining importance. (Boss et al., 1996b, 1996a; Hasegawa et al., 2001; Ban et al., 2003; Guo and Wang, 2010; Xie et al., 2011; Harel-Beja et al., 2019; Medda et al., 2021) The anthocyanin biosynthesis pathway has a structure that does not show much branching. Phenylalanine amonia lyase (PAL), chalconesyntase (CHS), chalconeisomerase (CHI), flavanone3-hydroxylase(F3H), anthocyanidinsynthase (ANS) and UDP are genes involved in the glucose-flavonoid 3-o-glucosyl transferase (UFGT) biosynthesis pathway. PAL is an important precursor gene because it is a precursor. First, phenylalanine is converted to trans-cinnamic acid by losing its ammonium via PAL. Trans-cinnamic acid is then converted to 4-coumarol-CoA via cinnamate 4-hydroxylase (C4H) and 4-coumate-CoA ligase (4CL). Then, one molecule of 4-coumarol-CoA and three molecules of malonyl CoA combine to form naringenin chalcone with the help of the CHS enzyme. Thereafter, naringenin chalcone rapidly forms naringenin by chalcone isomerase (CHI) After these reactions, naringenin is hydroxylated by flavanone 3-hydroxylase (F3H) enzyme to dihydroflavonols; dihydroflavonols are converted to leukoanthocyanidins by the enzyme dihydroflavonol 4-reductase (DFR). The ANS enzyme synthesizes anthocyanidins by using leukoanthocyanidins as a substrate. In the last step, anthocyanidins from anthocyanins by being glycosylated with sugar donors activated by UDP and activated by the UDPG-flavonoid-3-O-glucosyltransferase (UFGT) enzyme (Unal, et al., 2021; Xie et al., 2011). Although anthocyanins are found in very small amounts in plants, they have very important biological functions. They are highly functional in protecting the plant against plant pathogens, infections, high heat and UV light, regulating plant growth and regulating pollen growth (Boss et al., 1996b; Guo and Wang, 2010).

The most important biological activity of anthocyanins is their effects on reactive oxygen species (O2-superoxide, H2O2- hydrogen peroxide and OH-hydroxyl), which increase rapidly under various stress conditions (salinity, drought and low temperature) and cause cell damage. Anthocyanins provide biological protection against various cell damages by regulating reactive oxygen species (ROS) in the cell (Zaidi et al., 2019). They do this through their hydrogen donor abilities and by stimulating the antioxidant defense system (redox-sensitive nuclear factor erythroid 2-related factor 2 (Nrf2)-pathway) (Pahlke et al., 2021). Even very low doses of anthocyanins, which are mostly found in fruits and vegetables, fulfill very important functions in human health. They are indispensable sources of antioxidants due to their therapeutic effects against different types of cancer, eliminating the negative effects of free radicals, preventing chronic diseases. (Chusak et al., 2020; Speer et al., 2020; Tena et al., 2020; SPahlke et al., 2021; Yamuangmorn and Prom-u-Thai, 2021). Myrtle (Myrtus communis L.) is a plant species in the Myrtaceae family. It has high economic value due to its valuable secondary metabolites. Its leaves and fruit are rich in phenol and flavonoids. Its phytochemicals and high antioxidant capacity make myrtle plant more important day by day (Şimşek et al., 2017; Hennia et al., 2018; Aka Kaçar et al., 2020). In this study, the 'C-1' genotype with white fruits and the 'C-2' genotype with black fruits were used. The expression levels of PAL, CHS, CHI and F3H genes, which are important steps in anthocyanin biosynthesis, were investigated in the leaf and fruit tissues of these genotypes. In the study, anthocyanin biosynthesis, which causes coloration, was associated with the expression level of genes involved in the anthocyanin biosynthesis pathway.

Materials and Methods

Plant Material

Two different genotypes of the myrtle plant were used (Figure 1). Myrtle genotypes used in the study were taken from Çukurova University field. The 'C-1' genotype with white fruits and the 'C-2' genotype with black fruits were used in the study. Leaf and fruit tissues of the genotypes were harvested during fruit maturity. The tissues used in the study were taken from three different plants and frozen in liquid nitrogen and stored at -80°C until the analysis stage.

Total RNA Extraction and cDNA Synthesis

Total RNA extraction was performed with TRIzol reagent (TRI-Reagent). DNA contamination in samples was removed with DNase I (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The quality and integrity of the total RNA was checked with agarose gel electrophoresis and the NanoDrop 2000D. First-strand cDNA was synthesized from 2 µg of the RNA. Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (LOT:00756490) was used for cDNA synthesis. cDNA synthesis was performed according to the manufacturer's protocol. PCR cycling conditions determined in the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (LOT:00756490) were used for cDNA synthesis.

qRT-PCR For Expression Analysis

Primers of *PAL*, *CHS*, *CHI* and *F3H* genes were used for qRT-PCR (Table 1). Primers used in the study Ben-Simhon et. al. (2015) was taken from the study conducted. Three biological replicates were carried out and triplicate quantitative assays for each replicate were performed using SensiFAST SYBR Lo-ROX Kit (Cat. No.BIO-94020).

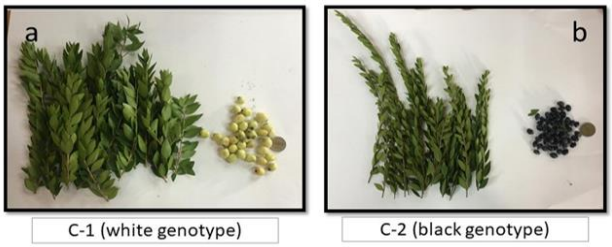

Figure 1. a) Image of leaf and fruit tissues of C-1 genotype with white fruits b) Image of leaf and fruit tissues of C-2 genotype with black fruits

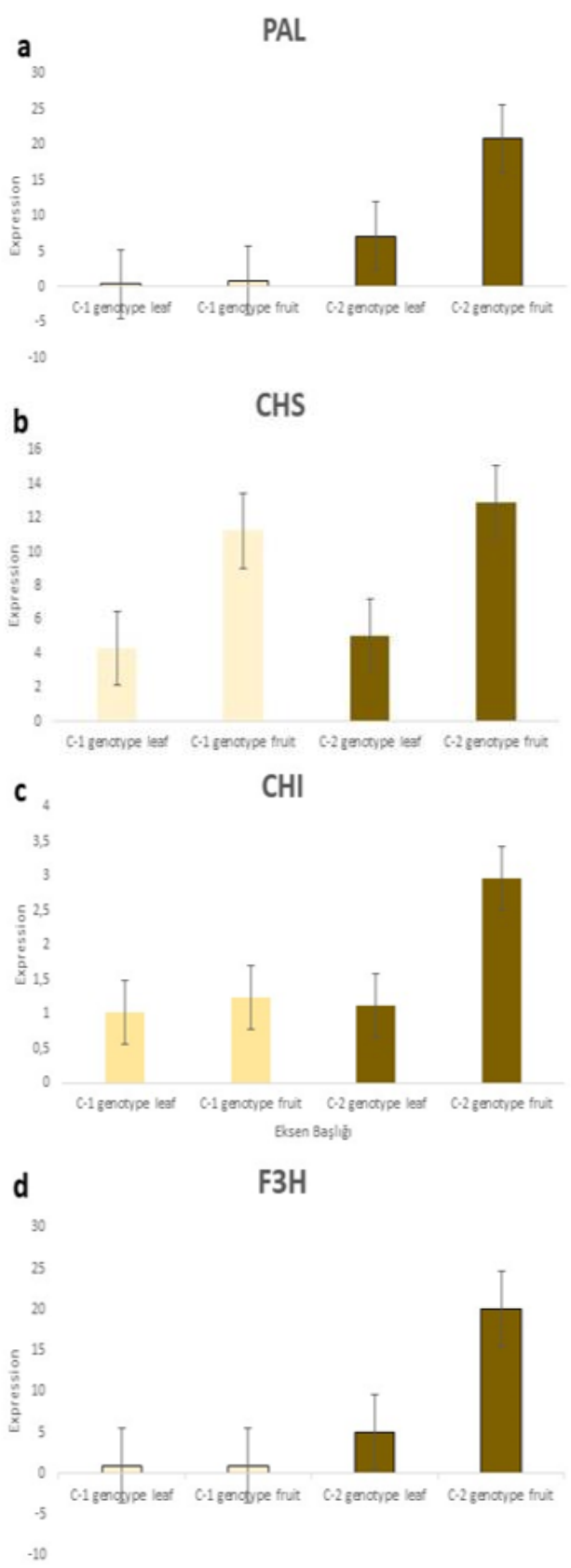

Figure 2. Expression levels of *PAL*, *CHS*, *CHI* and *F3H* genes in C-1 and C-2 genotypes.

The reaction volume was comprised of 10 µL containing 5 µL SYBER Green PCR Master Mix (Applied Biosystems), 0.8 Mm primer (forward and reverse) and 3 µL cDNA. Standard cycling conditions were 95°C for 10 min followed by 35–40 cycles of 95 C (15 s), 55–60°C (30 s) and 72°C (30 s) with a final extension at 95°C for (15 s), 60 C 1 min and 95°C for (15 s). The RPSII (Ribosomal protein S) gene was used as an internal control. The relative gene expression level was calculated using the formula $2^{-\Delta CT}$. At the same time, the standard errors of mean among replicates were calculated.

Result and Discusion

Expression levels of some genes related to anthocyanin biosynthesis were determined in myrtle plants with different colored fruits. The results of the analysis show that the expression level of genes associated with anthocyanin biosynthesis is higher in the C-2 genotype with black fruits compared to the C-1 genotype with white fruits (Figure 2-c). When the analysis results are examined, it is seen that the expression level of the *PAL* gene is quite high in the leaf and fruit tissues of the C-2 genotype (Figure 2-a). It was determined that the gene expression level was quite high especially in the fruit tissue of the C-2 genotype. On the other hand, *PAL* gene expression level remained very low in leaf and fruit tissues of C-1 (white) genotype. *CHS* gene expression level does not differ in the leaf and fruit tissues of the two genotypes and it creates a similar profile in terms of gene expression level. Although both genotypes have similar gene expression profiles, *CHS* gene expression level was found to be higher in fruit tissues compared to leaf tissues of C-1 and C-2 genotypes. When the *CHI* gene expression level was examined, it was determined that there was no significant difference in gene expression level in the leaf and fruit tissues of the C-1 (white) genotype, but there were significant differences in gene expression between the leaf and fruit tissues of the C-2 (black) genotype. *CHI* gene expression level is observed to be quite high in fruit tissue of the C-2 (black) genotype (Figure 2-c). The *F3H* gene expression profile is very similar to the *PAL* gene expression profile. It was determined that the expression level of *F3H* gene in the leaf and fruit tissues of the C-1 (white) genotype remained quite low, whereas the level of gene expression was quite high in the leaf and fruit tissues of the C-2 (black) genotype. Among the tissues of the C-2 (black) genotype, the *F3H* gene expression level was found to be higher in fruit tissue compared to leaf tissue (Figure 2-d).

Phenylalanine is a precursor required for the synthesis of anthocyanidins and the conversion of phenylalanine to anthocyanidins occurs as a result of a series of enzymatic reactions. (Xie et al., 2011). Studies are reporting that the expression level of *PAL*, *CHS*, *CHIi F3H*, *ANS* and UFGT genes, which are involved in anthocyanin biosynthesis, correlate with anthocyanin accumulation in plant tissues (Boss et al., 1996b, 1996a, 1996b; Guo and Wang, 2010; Rouholamin et al., 2015; Ben-Simhon et al., 2015; Harel-Beja et al., 2019; Medda et al., 2021;). Studies are reporting that the expression profile of genes associated with anthocyanin biosynthesis changes in different plant species (Xie et al., 2011). The *PAL* gene is a critically important gene for the phenylpropanoid pathway. The phenylpropanoid pathway is one of the pathways in which important polyphenolic metabolites are synthesized. Lignins, flavonoids, anthocyanins are the products of this pathway. The *PAL* gene is the starting element of this pathway and is also one of the enzymes that direct primary metabolites to secondary metabolite channels.

Table 1. Genes used in qRT-PCR Reactions

Gene	GenBank accession number	Forward Primer Sequence	Rewers Primer Sequence
PAL	Ben-Simhon et. al. (2015)	5'-TGGAGTGTCTCAGCAGTTGG-3′	5'-CGGAACAGCATAGGATGGAT-3′
CHS	Ben-Simhon et. al. (2015)	5'-CCCACTAAAGCGACCCATT-3′	5'-AGACCACAAAATGCCTCCAC-3′
CHI	Ben-simhon et. al. (2015)	5'-ACTACCATTGACGGGTGCTC-3′	5'-GTGTAAGTGCCCACGGATTT-3′
F3'H	Ben-Simhon et. al. (2015)	5'-CGAGTTGATACCGTTTGGG-3′	5'-GTTCAGCTTCTCGGGCAT-3′.
PgRPSII	Ben-Simhon et. al. (2015)	5'TCAATTTGTGAGGGTCGTTCT-3′	5'-TTCAAGAGTAGTAACCGATTCCA-3′

The PAL gene is not only associated with the phenylpropanoid pathway, but it has also been reported to be active during developmental periods and in various ecological conditions and to have inducing activity under some stress conditions (Yu et al., 2018). It has been reported that the expression level of the *PAL* gene differs in different plant species and tissues of these species. It has been emphasized that this difference is especially in plant development stages or in situations related to the biosynthesis of phenolic compounds (Yan et al., 2019). Most of the studies investigating the relationship of *PAL* gene with anthocyanin biosynthesis and pigmentation reported that anthocyanin accumulation in dark tissues and *PAL* gene expression level correlated (Boss et al., 1996b; Guo and Wang, 2010; Harel-Beja et al., 2019). When the results we obtained were examined, it was observed that the *PAL* gene expression level was high in the leaf and fruit tissues in the C-2 (black) genotype, while it was very low in the leaf and fruit tissues in the C-1 genotype. When the results obtained are evaluated, it is seen that the pigmentation and *PAL* gene expression level show parallelism (Figure 2-a).

In the study using grape varieties with different phenotypic characteristics, the relationship between anthocyanin accumulation and gene expression level was investigated. In the study, it was reported that the expression level of *PAL* gene was at the highest level in the grape variety with red color, whereas it remained very low in the grape variety with white fruit (Boss et al., 1996b). In another study comparing anthocyanin accumulation and antioxidant activity, mutant black rice and wild rice plants were used. It has been reported that anthocyanin accumulation is high in mutant rice grains with black color, and the expression level of *PAL, CHS,* and *CHI* genes is higher compared to wild plants (Zaidi et al., 2019).

Red color increases the market value of fruits and vegetables. In the study, which was carried out to clarify the red color formation in pomegranate and to increase the market value of the pomegranate, the genes associated with anthocyanin biosynthesis and the expression level of the genes involved in the regulation of these genes were examined. In the study, it was reported that the expression level of *PAL, CHS, CHI* and *F3H* genes was high in the variety with red fruits. In the same study, it was emphasized that the *DFR* gene, which regulates anthocyanin biosynthesis, was expressed at a high level in dark-colored fruit, but not in white-colored fruit (Rouholamin et al., 2015).

In addition to being the second important gene of the flavonoid pathway, the *CHS* gene also has functions such as protecting the plant against UV light and diseases and providing adaptation to various ecological conditions but the most important function of the *CHS* gene is that it is responsible for pigmentation. It has been reported that mutations are seen in the *CHS* gene in molecular studies performed on albino plants and therefore no pigment formation is observed (Durbin et al., 2000).

This clearly shows the effect of the *CHS* gene on pigment formation. When our data are examined, it is seen that the *CHS* gene expression level is high in tissues belonging to the C-2 (black) genotype. In the comparison between the tissues of the genotypes, it was determined that the expression level was higher in fruit tissues compared to leaf tissues (Figure 2-b).

Anthocyanin accumulation in flower tissues at different developmental stages and expression profile of genes involved in anthocyanin biosynthesis were investigated in *Malus hupehen.* In the study, it was emphasized that the expression level of *PAL, CHS, CHI* and *F3H* genes decreased in parallel with the decrease in the coloration rate. In the study, it was also stated that the expression levels of *CHS* and *CHI* gene were similar between tissues belonging to different developmental periods (Han et al., 2020). Similar results were obtained in our study and it was seen that *CHS* and *CHI* genes had similar profiles. *CHS* and *CHI* genes formed a different profile from the expression profile of *PAL* and *F3H* genes. It is seen that *PAL* and *F3H* genes form a similar profile, and there are high differences in gene expression levels between genotypes and tissues of genotypes however, it was determined that the expression profile of the *CHS* and *CHI* genes was similar and there were no major differences between genotypes. This suggests that *CHS* and *CHI* genes form a different profile than *PAL* and *F3H* genes, as they participate in the formation of different metabolites. There are studies reporting that *CHS* and *CHI* genes are involved in the biosynthesis reactions of different metabolites in the phenylproponoid pathway (Durbin et al., 2000; Xie et al., 2011). The *F3H* gene is a gene involved in the last steps in the anthocyanin biosynthesis pathway. Many studies are reporting that the *F3H* gene has an important function associated with anthocyanin accumulation in fruit tissues (Castellarin and Di Gaspero, 2007; Palapol et al., 2009; Feng et al., 2010; Niu et al., 2010). Castellarin et. al. (2007) reported that *F3H* gene expression level was correlated with anthocyanin accumulation and that as anthocyanin accumulation increased, *F3H* gene expression level also increased. The obtained dataare examined, it is seen that the expression level of the *F3H* gene belonging to the C-2 (black) genotype is quite high compared to the C-1 (white) genotype and it was observed that the expression level of the *F3H* gene was minimal in the C-1 genotype (Figure 2-d). At the same time, when the tissues of the C-2 (black) genotype were compared, it was determined that the *F3H* gene expression level of the fruit tissue was quite high compared to the leaf tissue. It is seen that the results we obtained in our study are in parallel with the literature data and that the *F3H* gene has a strong effect on anthocyanin accumulation. The data we obtained in our study determined that the expression level of genes in the anthocyanin

biosynthesis pathway is related to pigmentation, and increases in gene expression levels are also observed in parallel with the increase in coloration.

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