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Research Article

DETERMINATION OF EXPRESSION LEVELS OF GENES RELATED TO ANTHOCYANIN SYNTHESIS IN SOME POMEGRANATE GENOTYPES

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ABSTRACT. Pomegranate is a perennial deciduous fruit-bearing shrub with significant commercial value and a substantial place in cultural life. Pomegranate seeds are rich in phenolics and have various positive impacts on human health. Therefore, there is an ever-increasing interest in pomegranate and also in the consumption of fruits species with high anthocyanin contents. In the present study, expression levels of 4 different anthocyanin synthesis-related genes (CHS, CHI, F3H, PAL) in peel, seed and leaf tissues of 3 different pomegranate genotypes (33N53, İzmir-1479 and 19/147) were investigated with the use of qRT-PCR technique. Present analyses revealed that anthocyanin synthesis-related gene expression profiles were low in white genotype, medium in pink genotype and high in red genotype. In general, high expression levels were observed in CHS and CHI genes.

Keywords: *qRT-PCR, gene, RNA, CHS, CHI*

INTRODUCTION

Pomegranate is a perennial deciduous fruit-bearing shrub belonging to *Punica* genus of *Punicaceae* family. Besides commercial value, it has a significant place in cultural life. Cultural life of pomegranate goes back to a long way. It was reported in various resources that pomegranate culture went back to 5000 years ago. Hight of pomegranate shrubs vary between 2-5 m. Multi-seed fruits are composed of flesh seeds with colors ranging from white to dark red. Fruits are grouped as sweet, sour and tart in flavor and marketed accordingly. Since fruits are used in several productions, preferences may vary with the production industries. Pomegranate is originated in Middle East covering especially Iran and Southeast Turkey, Caucasus and Northern India. In several resources, origin of pomegranate is indicated as Iran, Caucasus and North India. Pomegranate has been known in Anatolia and entire Mediterranean basin for thousands of years. Today, pomegranate is cultivated over large areas ranging from Australia to South Africa, from the USA to China [1]. Pomegranate cultivating countries include the USA, Russia, Afghanistan, China, Morocco, Palestine, India, Iraq, Iran, Peru, Israel, Italy, Spain, Cyprus, Syria, Egypt, Saudi Arabia, Thailand, Tunisia, Turkey and some other countries [2]. World annual pomegranate production is around 3 million tons. India, Iran and Turkey are the leading producers. The pomegranate production of Turkey was 170.963

tons in 2009 and the value continuously increased and reached to 537.847 tons in 2018 [3].

Pomegranate seeds are rich in phenolics and have various positive impacts on human health. Therefore, there is an ever-increasing interest in pomegranate. Pomegranate prevents blood oxidation, increase hematopoietic cells and protects cardiovascular health. Pomegranate seeds are quite rich especially in iron, potassium and vitamin C. Anthocyanin, providing red color of the fruit, boosts the immune system. Antioxidants reduce infectious pathogen effect and increase the number of resistant cells. Pomegranate reduces the incidence of flue and upper respiratory track diseases. It also replenishes thrombocyte cells in blood, prevents edema and relieves rheumatism and join pains. Pomegranate regulates blood pressure and reduce anemia-related faints. With tonic effects, it also replenishes death cells of the skin. Since it is a strong antioxidant, pomegranate prevents proliferation of cancerous cells. It cleans stomach and intestines and removes them from the body. When consumed regularly, pomegranate supports regional weight loss and prevents kidney and bladder stone formations. Pomegranate plays a protective role against several diseases including cardiovascular diseases, Alzheimer, type-2 diabetes and obesity [4]. Pomegranate is also rich in anthocyanins, ellagitannins and the other phenolic substances with a proven antioxidant and antitumor activity [5]. Majority of these phenolics, especially the ones with high molecular weight, exist in skin section of the fruit [6]. Important phenolic compounds of pomegranate include anthocyanins, flavanol glycosides, procyanidins, ellagic acid and derivatives. High antioxidant capacity of the pomegranate comes from tannins, flavonoids and anthocyanins. With antioxidant activity of these secondary metabolites, pomegranate has important antimutagenic and anticancerogenic characteristics [7]. Anthocyanin means flower and blue in Latin. Anthocyanins are water-soluble natural pigments and are composed of anthocyanidins containing one or more sugars [8]. Flavylium ion is the building block of anthocyanidins isolated from the plants. Besides antioxidant activity and UV-protection, anthocyanidins play a significant role in defense, pollination and regeneration functions of the plants [9]. The anthocyanins existing in glycoside forms in cell cytoplasm are composed of some sugar and non-sugar (aglycon) substances. Aglycon part is composed of anthocyanidins. Anthocyanidins and number of position of sugars attached to them influence antioxidant activity of these substances [10, 11]. Various different genes play a role in anthocyanin biosynthesis. These genes include phenylalanine ammonium lyase (PAL), chalcone synthase (CHS) and anthocyanidin synthase (ANS). Phenylalanine is precursor of this synthesis and conversion from phenylalanine into anthocyanins is realized through enzyme-catalyzed reactions [12]. Anthocyanins are essential pigments with significant contributions to fruit quality. Therefore, the biosynthetic pathway to anthocyanins should be well-comprehended [8].

There is a great interest in the consumption of fruit species with high anthocyanin contents because of the highly significant effects of these substances on human health and nutrition. Pomegranate fruits are quite rich in anthocyanins. Therefore, this study was conducted with 3 different pomegranate genotypes to investigate the expression levels of 4 different anthocyanin synthesis-related genes in fruit skin, seed and leaf tissue with the use of qRT-PCR technique.

MATERIAL AND METHODS

Plant material

The genotypes used in this study were selected from Pomegranate Genetic Collection at Horticulture Department of Çukurova University Agricultural Faculty. Skin, seed and leaf tissues were taken from "33N53", "İzmir-1479" and "19/147" genotypes of this genetic collection.

Collection of tissue samples of the genotypes

To determine the expression levels of anthocyanin biosynthesis-related genes in 3 different pomegranate genotypes with different coloration levels, 3 different tissues were used at full-ripe stage. Genotype tissues were taken in October 2019. About 50 g tissue samples were taken, labeled, wrapped in aluminum foil and placed into liquid nitrogen. Tissue samples frozen in liquid nitrogen were stored at -80ºC until RNA isolation phase.

RNA extraction

For RNA isolation, the tissues stored at -80°C were used. Tissues were ground in a mortar with liquid nitrogen, taken (100 mg) into Eppendorf tubes and made ready for isolation. RNA isolation was performed in accordance with RNeasy Mini Kit (Qiagen) Purity and concentration of isolated RNA was measured with a NanoDrop spectrophotomer. RNA quality was assessed through the existence of 28S and 18S ribosomal RNA sub-units. Existence of RNA sub-units was determined with agarose gel electrophoresis. For this purpose, 1,5% agarose gel was prepared. The 5 µl of isolated RNA samples were supplemented with 1 µl 6X loading dye and 5 µl ddH_2O. Samples were then run under 100 volts for 60 min. At the end of this period, agarose gel was imaged under UV light.

qRT-PCR Analyses

Applied Biosystems High Capacity cDNA Reverse Transcription Kit was used in cDNA synthesis. Four different genes (CHS, CHI, F3H, PAL) known to be related to anthocyanin biosynthesis were used in Quantitative Real Time PCR processes. The PgRPSII gene was used as endogen control. The primers of relevant genes are provided in Table 1.

Quantitative Real-time PCR (qRT-PCR) was performed in an ABI 7500 real-time system. The 25 µl reaction system contained 1 µl of diluted cDNA, 11.25 µl of Fast SYBR™ Green Master Mix (ThermoFisher Scientific), and 0.5 µl of each primer. The cycling parameters were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 35 s. The PgRPSII control gene was used for the normalization of the CT / CP values of the target gene. The qRT-PCR results were calculated by comparative Ct method. In this method, the average Ct values of all samples are calculated. The ΔCT and ΔΔCT values were then calculated and the results were evaluated using the 2- ΔΔCT formula. It has been determined at which levels anthocyanin-related genes are expressed relative to control genes.

Table 1. *Information about the genes used in this study [13]*

Genes	Forward Primer	Reverse Primer
PAL	5'-TGGAGTGTCTCAGCAGTTGG-3′	5'-CGGAACAGCATAGGATGGAT-3′
CHS	5'-CCCACTAAAGCGACCCATT-3′	5'-AGACCACAAAATGCCTCCAC-3′
CHI	5'-ACTACCATTGACGGGTGCTC-3′	5'-GTGTAAGTGCCCACGGATTT-3′
F3'H	5'-CGAGTTGATACCGTTTGGG-3′	5'-GTTCAGCTTCTCGGGCAT-3′.
PgRPSII	5'TCAATTTGTGAGGGTCGTTCT-3′	5'-GATTCAAGAGTAGTAACCGATTCCA-3′

RESULTS AND DISCUSSION

Three different pomegranate genotypes were selected from 90 pomegranate (*P. granatum*) genotypes in Pomegranate Genetic Collection at Horticulture Department of Çukurova University Agricultural Faculty and expression levels of 4 anthocyanin synthesis-related genes in fruit skin, seed and leaf tissues were investigated with qRT-PCR technique.

Tissue expression levels of "33N53" genotype with white seed and green skin color, "İzmir-1479" genotype with pink skin and seed color and "19/147" genotype with dark red skin and seed color were analyzed.

Expression levels of relevant genes were determined with Real time PCR analyses. The 2-ΔΔCt formula was used in analyses and gene expression levels of the genotype tissues are presented in Fig. 1-4.

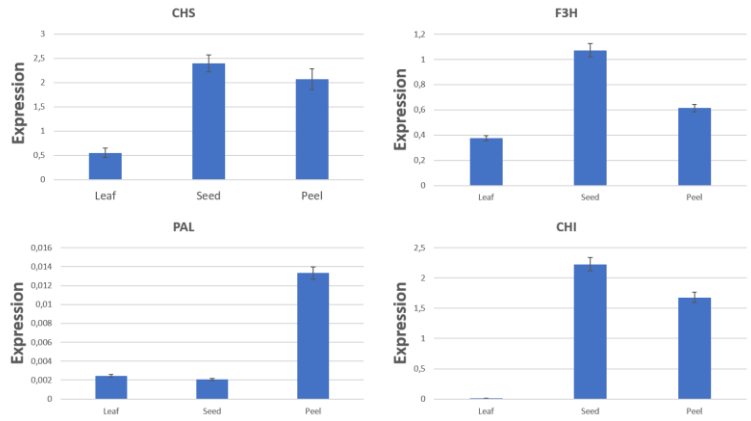

Fig. 1. *Expression levels of genes in tissues of İzmir-1479 genotype*

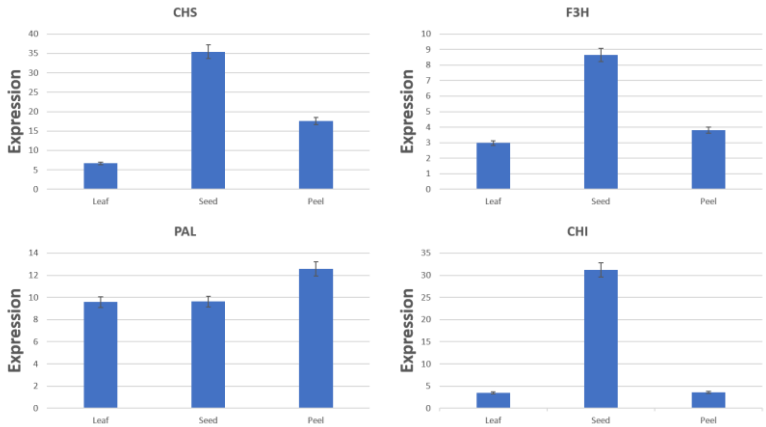

Fig. 2. *Expression levels of genes in tissues of 19/147 genotype*

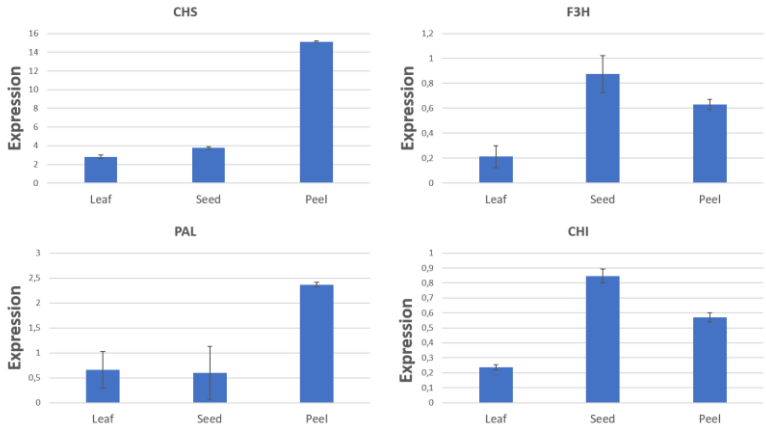

Fig. 3. *Expression levels of genes in tissues of 33N53 genotype*

With regard to CHS gene expression levels of 33N53 genotype, the lowest expression was observed in leaf tissue. Expression level was 3 in leaf tissue and 15 in skin tissue. The greatest expression level was observed in skin tissue which was 3 times as much of leaf tissue. Considering the CHI gene expression levels, the lowest expression level was observed again in leaf and the greatest expression level was observed in seed tissue, which was 3 times as much of leaf tissue.

With regard to F3H gene expression levels of 33N53 genotype, the lowest expression level was observed in leaf tissue and the greatest expression level was observed in seed tissue, which was 4 times as much of leaf tissue. Considering the PAL gene expression levels, the lowest expression levels were observed in leaf and seed tissues and the greatest expression level was observed in skin tissues, which was almost 5 times as much of leaf and seed tissues. Present analyses revealed that in 33N53 genotype, increases were observed in expression levels of CHS and PAL genes in skin tissue and CHI and F3H genes in seed tissue. Despite white seed and green skin color, slight increases were observed in expression levels of anthocyanin biosynthesis-related genes. Zhao et al. [14] reported insignificant differences in expression levels of anthocyanin synthesis-related genes of white-color pomegranate fruits. However, differences were observed in expression levels of relevant genes. Such differences were mainly attributed to differences in genotypes and tissues. High increases especially in expression levels of CHS gene were because CHS is the key trigger gene of flavonoid pathway. CHS and CHI are the key trigger genes of flavonoid pathway. Following the catalyzing of CHI gene, flavonoid pathway divided into two pathways responsible for polyphenol and anthocyanin biosynthesis [15]. Therefore, is was thought that the differences in expression levels of CHS or CHI genes were not solely related to anthocyanin biosynthesis.

Considering the expression levels of CHS gene in different tissues of İzmir-1479 genotype, the lowest expression level was observed in leaf tissue and the greatest expression levels was observed in skin tissues, which was 5 times as much of leaf tissue. For expression levels of CHI gene, the lowest expression level was observed in leaf tissue (0.1) and the greatest expression level was observed in seed tissue (2,3), which was 23 times as much of leaf tissue.

With regard to expression levels of F3H gene in different tissue of İzmir-1479 genotype, the lowest expression level was observed in leaf tissue and the greatest

expression level was observed in seed tissue, which was 3 times as much of leaf tissue. Considering the expression levels of PAL gene, the lowest values were observed in leaf and seed tissues and the greatest value was observed in skin tissue, which was 6.5 times as much of leaf tissue. In İzmir-1479 genotype with pink seed and skin color, expression levels of CHS, CHI and F3H genes were high in seed tissue and expression level of PAL gene was high in skin tissue. Although CHS and CHI genes catalase flavonoid pathway, F3H and PAL genes are responsible for activation of pathway in anthocyanin biosynthesis mechanism after CHI genes. Therefore, expression profile of F3H and PAL genes is relative lower than the expression levels of CHS gene.

F3H is an important enzyme on biosynthetic pathway of plant flavonoids. In plants, F3H gene encodes flavonoid biosynthesis pathway, in other words flavanone 3-hydroxilase enzyme [16], PAL gene catalyzes the first and rate-limiting step in phenylpropanoid pathway, such a case then provides precursors for various secondary metabolites including flavonoids [17] and plays critical roles in plant secondary metabolism which is an important issue for the interactions between the plants and the environment. Decreases might have been seen in expression levels just to catalyze different secondary metabolite pathways.

Considering the expression levels of CHS gene in different tissues of 19/147 genotype, the lowest value was observed in leaf tissue (5) and the greatest value was observed in seed tissue (35), which was 7 times as much of leaf tissue. For expression levels of CHI gene, the lowest value was observed in leaf tissue and the greatest value was observed in seed tissue, which was 10 times as much of leaf tissue.

Considering the expression levels of F3H gene in different tissues of 19/147 genotype, the lowest value was observed in leaf tissue (3) and the greatest values was observed in seed tissue, which was 3 times as much of leaf tissue. For expression levels of PAL gene, the values were observed in lead and seed tissue and the greatest value was observed in skin tissue, which was 1.5 times as much of leaf tissue. In 19/147 genotype with dark red seed and skin color, expression levels of CHS, CHI and F3H genes were high in seed tissue, expression levels of PAL gene were high in skin tissue. Based on expression levels, the greatest anthocyanin content was observed in seeds of CHS gene of 19/147 genotype and the lowest anthocyanin content was observed in leaves of CHI gene of 33N53 genotype. Gene anthocyanin contents were expressed at quite high levels in red-color genotypes. In some genes, higher expression levels were also achieved in seeds. Such a case may be considered as the reason of differences in anthocyanin contents of genotypes and tissues.

Two types pf genes play an important role in anthocyanin biosynthesis. These are structural genes and regulatory genes (transcription factors) controlling the expression levels of structural genes [18]. Changes in color tones may be resulted from differences in expression levels structural and regulatory genes.

CHI is the key enzyme of phenylalanine metabolism pathway providing various flavonoids. It play an important role in pigment synthesis, flower, fruit and seed color formation [19]. CHS represent the beginning of specific flavonoid pathway and is responsible for formation of a chalcone from one Þ-coumaroyl-CoA and three malonyl-CoA molecules to generate two phenyl rings of flavonoid skeleton (C6-C3-C6) [15]. CHS is an important enzyme catalyzing the first stable step of flavonoid biosynthetic pathway [20]. Therefore, expression levels of these genes are not directly related to anthocyanin biosynthesis, but they are responsible for catalyzation of the other genes with an important function in anthocyanin biosynthesis pathway. Then, changes may be seen in expression

levels. F3H is also an important enzyme in biosynthetic pathway of plant flavonoids. In plants, F3H gene encodes flavanone 3-hydroxilasine enzyme of flavonoid biosynthesis pathway [17].

Zhao et al. [14] measured the transcript levels of the genes related to anthocyanin biosynthesis in red and white fruits quantitatively with real time PCR. Anthocyanin was not encountered in white genotypes since lack of ANS expression may be the primary factor responsible for inexistence of anthocyanins in white pomegranates. Except for pgANS, white pomegranate skins were containing transcripts of all defined genes. Therefore, lack of pgANS expression may be the primary factor responsible for inexistence of anthocyanins in white pomegranate. Harel-Beja et al. [21] investigated anthocyanin and punicalagin contents of pomegranates and determined gene expression and metabolite (anthocyanins and punicalagin) accumulation in three growth stages. Researchers determined high and low anthocyanins respectively in skins of red and pink genotypes. Red and pink pomegranate cultivars exhibited the greatest difference in gene expression during the transition from early fruit development stage to late fruit development stage. In other studies with different fruits, similarly high anthocyanin contents were reported for red cultivars. Wei et al. [22] investigated the expression levels of F3H, DFR, ANS and UFGT genes in litchi fruits and reported that expression levels in fruit pericarps were quite weak in non-red cultivars as compared to red ones. Karanjalker et al. [23] investigated gene expression levels in skins of green, yellow and red-color mango fruits and reported greater anthocyanin contents in red-color cultivars. Palapol et al. [24] conducted a study with fast-coloring mangosteen fruits and investigated expression levels of GmMYB10 gene both in ripening and post-ripening stages with the use RT-PCR method. Researchers reported increasing expression levels with the progress of coloration and indicated that this gene promoted anthocyanin biosynthesis. Feng et al. [25] determined expression levels of PyMYB10 gene in pear leaves, flowers and fruits with the use of RT-PCR method and reported greater expression levels of PyMYB10 gene in red tissues than the non-red ones.

CONCLUSION

In present study, expression levels of 4 different anthocyanin synthesis-related genes (CHS, CHI, F3H, PAL) in fruit skin, seed and leaf tissues of 3 different pomegranate genotypes (33N53, İzmir-1479 and 19/147) were investigated with the use of qRT-PCR technique. Present analyses revealed that anthocyanin synthesis-related gene expression profiles were low in white genotype, medium in pink genotype and high in red genotype. In general, high expression levels were observed in CHS and CHI genes because of formation after division in anthocyanin synthesis pathway. Although the expression levels of the other genes seemed to be low, expression level of F3H gene was also high in red genotype. It can be stated that free radical-neutralizing antioxidant effect was greater in red fruits. Therefore, there is a growing interest in consumption of red fruits with an important place in human health. Such a case then directed researchers to breeding of fruit species with a rich nutritional composition [26]. As it was seen in present study, high gene expression levels in red fruits could be related to rich nutritional composition and such high expression levels could be used in material selection of breeding programs. Consumption, thus production quantities of fruit species with a rich nutritional composition are continuously increasing. Fruit color is formed based on plant genetic

structure, biotic and abiotic stress factors in growing environment and color is among the most important quality parameters influencing consumer preferences.

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