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Chalcone Isomerase Gene Expression in Different Stages of *Petunia Hybrida* Flowering and Various Flower Colors

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Abstract

Petunia (*Petunia hybrida*) is one of the important plants in horticultural industry. This plant is being used as a model ornamental plant and is one of the most important plants in floriculture market. Flavonoids are the main pigment in this plant. So, genetic engineering with the goal of color alteration in petunia is focused on flavonoids. To gain a global perspective on genes differentially expressed in petunia's flowers pigment pathway, we investigated the expression of chalcone isomerase (*chi*) as an essential gene in biosynthesis pathway of pigment production in different types of flower color of petunia and various stages of flowering in this plant. Also, we measured the concentration of total flavonoids, anthocyanins and naringenin to evaluate the probably relationship between the expression profile of *chi* gene and the concentration of mentioned pigments. The results indicated that chalcone isomerase expression had different profile in different petal color of *P. hybrida*. So that, the most *chi* expression observed in red petunia flowers. Naringenin concentrations was the most value in this color flower. In comparison of flowering stages, stage 1, had the most expression. In other words, when the flowers fully closed (bud stage), *chi* expression and concentration was in the highest value. Our results showed that *chi* is a key gene in pigment biosynthesis pathway so that in absence of this gene, pigment pathway will be stopped. Identification of effective genes in different pathways of secondary metabolite production will assist in accurate selection of genes for genetically modification of pathways and production of various metabolites.

Keywords: Chalcone isomerase, Naringenin, *Petunia hybrida*, Pigment pathway, Real-time PCR.

INTRODUCTION

Flower color is one of the most noteworthy characteristics in ornamental plant breeding. Flavonoids, a class of low-weight phenolic flower color related compounds, are major pigments which accumulate in vacuoles (Chen *et al*., 2017). They are derived from the general phenylpropanoids pathway. So far, more than 9,000 flavonoids have been identified. They are classified into many subgroups such as chalcones, flavones, flavonols, flavandiols and anthocyanins according to the degree of oxidation and saturation of the central pyran ring (Freyre and Wilson, 2014). Anthocyanins are members of flavonoids responsible for a range of pigments from red and orange to blue and purple. In fact, the color of anthocyanin pigments is determined by hydroxylation pattern of their B-ring (Fig. 1) (He *et al*., 2013), one hydroxy group leads to colors ranging from orange to bright red, two hydroxy groups lead to dark red to magenta and three hydroxy groups produce violet to blue color (Voorhuijzen *et al*., 2020).

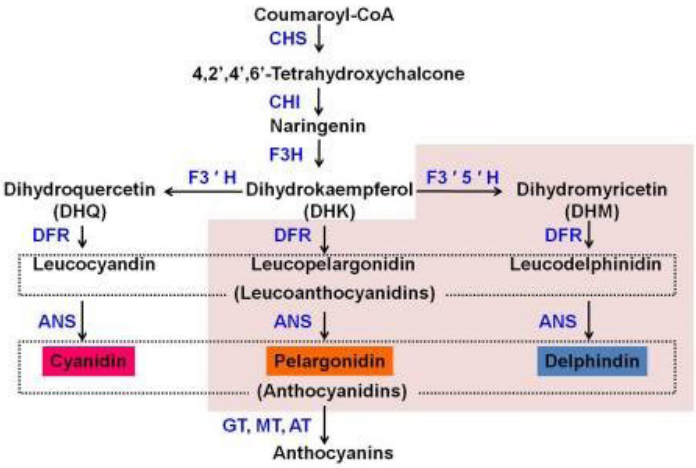

Fig. 1. The flavonoids biosynthesis pathway leading to the synthesis of pigments.

As is shown in Fig. 1, early steps required for the synthesis of all flavonoids is catalyzed by chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone-3-hydroxylase (F3H). The late steps of anthocyanin and proanthocyanidin biosynthesis are mediated by dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose: flavonoid-3-O-glucosyltransferase (3GT). In fact, anthocyanidins which are the central chromophores of anthocyanins are produced by enzymes from CHS to ANS. 3-glucosides that are the first stable anthocyanin are produced by glucosylating the 3 position of anthocyanidins by 3GT. Further glycosylation and acylation to anthocyanidin 3-glucosides, occur in a species-specific manner. This specify along with the pH of the vacuole's matrix and the vacuolar lumen are the most important factors in the variety of flower color (Bashandy and Teeri, 2017) because anthocyanins have a reversible structure dependent on the pH of their solvent, which by changing their structure their absorption spectrum changes drastically. The activity of H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (PPase) on the tonoplast, acidify vacuolar lumen (He *et al*., 2013; Ohmiya *et al*., 2014) and normally in an acidic vacuole, anthocyanins show reddish to purplish colors. They would change to bluish colors in a basic vacuole (Haselmair-Gosch *et al*., 2018). Epidermal cell shapes, co-pigments and the expression levels of genes regulating the flavonoid and anthocyanin biosynthesis also affect flower color (Okitsu *et al*., 2018). Therefore, investigation of differentially expressed genes from different-colored flowers and evaluating their relation to concentration of some pigments seems to be essential.

Among the genes and enzymes identified in the flavonoid pathway, chalcone isomerase (CHI) enzyme catalyzes the isomerization of naringenin chalcone into the corresponding flavanone

(Fig. 1) (Keykha Akhar *et al*., 2016; Noda *et al*., 2017). This enzyme belongs to the family of isomerases, specifically the class of intramolecular lyases. Chalcone isomerase has a core 2-layer alpha/beta structure. It has recently come into focus because of its involvement in the stress response and pigment production (Nakatsuka *et al*., 2013; Wang *et al*., 2018). For example, some plant species such as carnation, China aster and cyclamen, are known to accumulate chalcones, and show yellow pigmentation in the flowers by a reduction in CHI activity (Nishihara *et al*., 2005). Understanding mechanisms lead to pigmentation of flowers is the first step for manipulation the flower color which is commercially valuable especially in ornamental plants. In this study, to evaluate the probable relationship between the expression of *chi* gene and the concentration of total flavonoids, total anthocyanins and naringenin, four distinct colors (red, blue, pink and white) of *P. hybrida* were selected and evaluated in different stages of flowering.

MATERIALS AND METHODS

Plant materials

Plants of *P. hybrida* with four flower petals colors of white, pink, red and blue, were grown under standard greenhouse conditions (16-17 °C night temperature / 21-24 °C day temperature and photoperiod 16/8 (light/dark)). Petunia's flowers were collected in 3 different stages and immediately immersed in liquid nitrogen after excision and preserved in a -80°C ultra-low temperature freezer until RNA extraction. Evaluated flower stages for *chi* expression were: 1) closed flower's bud with 1 cm length; 2) semi-closed flower's bud with 3 cm length; and 3) fully opened flowers (Fig. 2). Simultaneously, the same petal tissues were gathered to measure the mentioned pigments content.

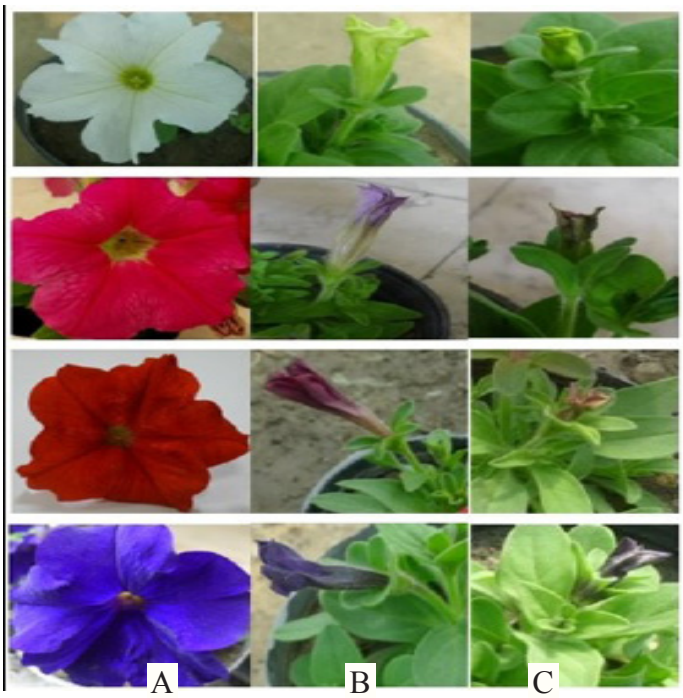

Fig. 2. Different stages of flowering in 4 colors of *Petunia* (from top to down, white, pink, red and blue). A) Stage 3: Flowers are fully-opened; B) Stage 2: Semi-closed flower's bud with 3 cm length; C) Stage 3: Closed flower's bud with 1 cm length.

RNA extraction and cDNA synthesis

Total RNA was extracted separately from four colors (white, pink, red and blue) of petunia in 3 stages of flowering using Denazist Column RNA Isolation Kit (#S-1020, Iran). RNA integrity

was confirmed by 1% agarose gel electrophoresis. After treating with DNase I (Thermo Scientific #EN0525, USA) at 37 °C for 30 min to remove probable DNA residues, RNA concentration was measured using a Nanodrop spectrophotometer. Synthesis of first strand cDNAs was carried out using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1622) following the manufacturer's protocol.

Quantitative real-time PCR

To perform the real-time quantitative PCR (qrtPCR), primers for the amplification of *chi* gene were designed to amplify 137 bp fragment (Table 1). The qrtPCR was carried out with the SYBR® Premix Ex Taq TM II kit (TaKaRa #RR820L). Each reaction contained 2 µL of the first-strand cDNA as template, in a total volume of 20 µL reaction mixture. The amplification program was performed as 95°C/10 min followed by 95°C/15 sec, 60°C/15 sec and 72ºC/30 sec (40 cycles). In order to normalize the qPCR data, elongation factor (*ef1A*) was selected as housekeeping gene and the following specific primers with product size of 180 bp were designed and used (Table 1). The experiments were repeated three times on independently isolated mRNA preparation as biological repeats. To increase the reliability of gene expression analysis, real-time PCR experiments were done with two identical technical replications. The accuracy of qrtPCR reactions were confirmed using melting curves for the products at the end of each run. The calculation of relative gene expression was done based on methods that explain expression ratio equal to $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001) while the white color flowers were employed as control samples.

Table1. Sequences of primers used for the amplification of *chi* and *ef1A* genes in *P. hybrida*.

Elongation factor (*ef1 A*)	**Chalcone isomerase (*chi*)**	**Primers/Genes**
CGGCGTCAACACCTACACC	TCTCCTCCAGTGTCCGTTAC	Forward Primer (5'→3')
GAAGTTTCCTGCTGCGATGG	ACAAACTTCCCTTCTATCTCCAG	Reverse Primer (5'→3')
180	137	Length of Fragment

Measurement of total flavonoids, total anthocyanins and naringenin using absorption spectra

Flavonoid extraction

Pigments were extracted from the petals by homogenizing 2 g of the petal tissue in 3 ml ethanol containing 0.1% HCl. The content of 300 µl of each pigment's extracts were loaded on the Elisa reader plates of nanodrop. Absorption spectra were determined at 415 nm for total flavonoids, 535 nm for total anthocyanins and 290 nm for naringenin (Fig. 3). Conversion naringenin data to g was done with standard curve.

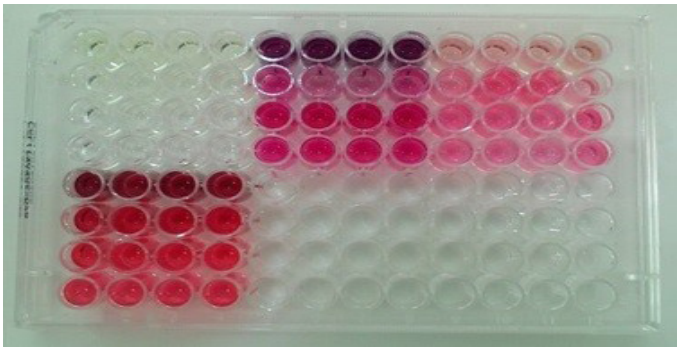

Fig. 3. Extracted flavonoids from petunia's petals in different stages of flowering with 4 colors and measurement of flavonoids, anthocyanins and naringenin with nanodrop.

Statistical analysis

This experiment was performed based on a completely randomized design with three (qrtPCR) and four (petal pigment concentration measurement) biological replications in the samplings. To increase the reliability of gene expression analysis, real-time PCR experiments were also done with two identical technical replications. The statistical analyzes of the data were done using T-student and Duncan's range test ($\alpha < 0.05$; SPSS v.16).

RESULTS

In this research, chalcone isomerase (*chi*) gene expression was investigated in four *P. hybrida* colors (white, blue, pink, and red) in three different stages of flowering as the key gene in pigment production biosynthesis pathway. The white flowers were selected as control samples.

The highest level of *chi* expression in closed bud stage was observed in red flowers with 6.1 times more than white flowers. In blue and pink flowers, the expression of *chi* gene had increased up to 5.9 and 5.2 times, in compare to the white color ones (Fig. 4).

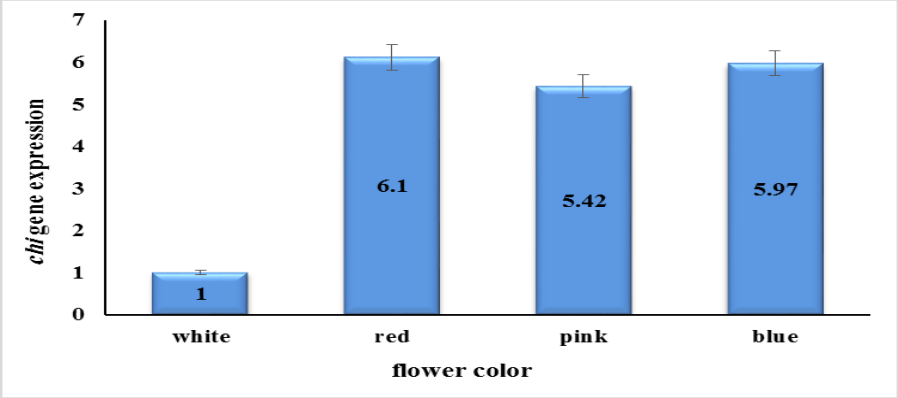

Fig. 4. The *chi* gene expression in 4 flower colors of petunia (bud flowers with 1 cm length) in compare to the control samples (white flowers).

Chalcone isomerase enzyme produces naringenin. Therefore, which stage, absorption of naringenin was measured at 290 nm and the values were converted to concentrations (µg/ml) using standard curve. The highest concentration of naringenin was observed in red flowers which was 1.9 times the control samples (white flowers) whereas, in blue and pink flowers, it was 1.2 and 1.07 times more than control samples respectively (Table 2).

Table 2. Total flavonoids (F), anthocyanins (A) and naringenin (N) in 4 colors of petunia and 3 stages of flowering using absorption spectra at 415, 535 and 290 nm wave lengths, respectively.

	Stage 1 of flowering			Stage 2 of flowering			Stage 3 of flowering		
Color	F	A	N	F	A	N	F	A	N
		(ml/µg)			(ml/µg)			(ml/µg)	
White	0.39d	0.24d	17.63c	0.60d	0.37d	14.41c	0.85d	0.42d	10.88c
Red	9.32a	5.31a	34.45a	11.59a	7.34s	31.87a	14.37a	11.19a	29.40a
Blue	6.69b	3.94b	22.33b	8.00b	5.50b	18.91b	11.12b	10.22b	16.28b
Pink	2.28c	2.32c	19.04bc	3.84c	4.66c	15.46c	5.95c	8.82c	11.68c

*In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan's range test.

In the second sampling stage (semi-closed bud flowers with 3 cm length), *chi* expression in red flowers was 4.8 times more than the control samples, this was the highest expression ratio among studied colors of petunia. In blue and pink flowers, the expression ratio of chi gene were 4

and 3.8 times more than control samples (white color ones) (Fig. 5). In addition, naringenin concentration in red, blue, pink and white flowers was 31.87, 18.91, 15.46 and 14.41 µg/ml respectively. In this stage, in red flowers, flavonoids and anthocyanins were 19 times more than control (Table 2).

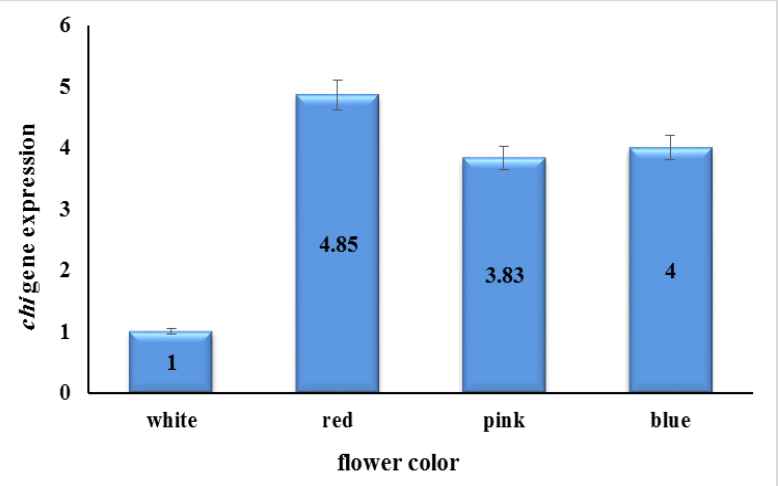

Fig. 5. The *chi* gene expression in semi-closed bud flowers stages in petunia's flowers in compare to the control samples.

When the flowers completely opened (stage 3), the highest *chi* expression was observed in red flowers (2.8 times in compare to white flowers). The *chi* gene expression in blue and pink flowers increased to 2.7 and 2.5 times the control samples (Fig. 6). In red flowers, total flavonoids, anthocyanins and naringenin were also more than white flowers (16.9, 26.6 and 2.7 times, respectively) (Table 2).

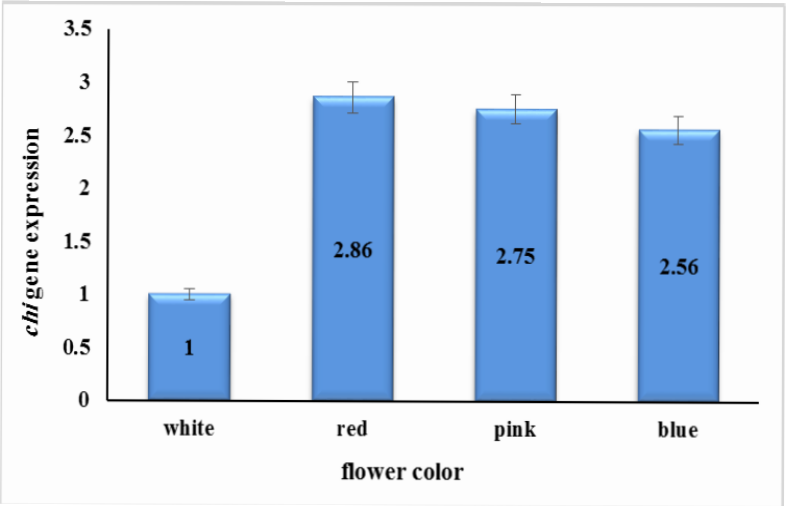

Fig. 6. The *chi* gene expression in fully-opened flowers stages in compare to the control samples.

In order to compare *chi* expression in different stages of flowering (independent of flower color) in petunia, fully-opened flowers stage (stage 3) was selected as the control. In stage 1 (closed flower's buds with 1 cm length) the highest *chi* expression ratio was observed (Fig. 7). The most naringenin concentration in all evaluated colors, was also observed in this stage (Table 3).

Also, increase the naringenin concentration was observed in stage 1 (Table 3) and this stage had significant difference with other stages ($\alpha<0.05$). Whereas total flavonoids and anthocyanins were in maximum amounts in fully-opened flowers stage (stage 3) (Table 3).

Table 3. Total flavonoids, anthocyanins and naringenin in 3 stages of flowering in petunia plants using absorption spectra.

	Flavonoid			Anthocyanin			Naringenin		
Color	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 1**	**Stage 2**	**Stage 3**
White	0.39c	0.60b	0.85a	0.24c	0.37b	0.42a	17.63a	14.41b	10.88c
Red	9.32c	11.52b	14.37a	5.31c	7.34b	11.19a	34.45a	31.87a	29.4a
Blue	5.35b	8.00b	11.12a	3.94c	5.50b	10.22a	22.33a	18.91b	16.28c
Pink	2.28c	3.84b	5.95a	2.32c	4.66b	8.82	19.04a	15.46b	11.68c

*In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan's range test.

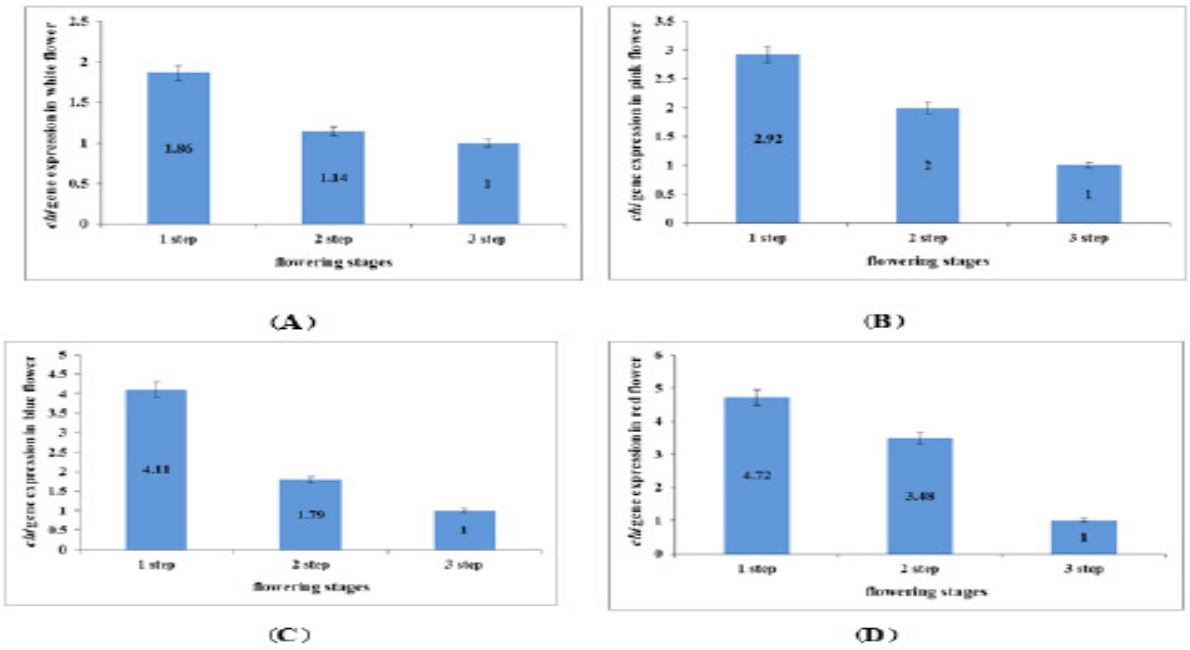

Fig. 7. The *chi* gene expression in different stages of flowering (Stage 1: Closed flower's bud with 1cm length; Stage 2: Semi-closed flower's bud with 3 cm length; Stage 3: Fully-opened flower) in compare to control (stage 3) in petunia's flowers with different colors. A) White flowers; B) Pink flowers; C) Blue flowers; D) Red flowers.

DISCUSSION

Flavonoid pigments are responsible for flower color and anthocyanins are a major class of flavonoids that produce the blue, violet, pink and red colors of flowers and other tissues. In fact, CHS begins the formation of chalcones which initiates the biosynthesis of anthocyanin, then chalcone is converted into naringenin by CHI, and then flavanone-3-hydroxylase (F3H) hydroxylates the 3rd positions of Naringenin's central ring which leads to the production of dihydrokaempferol (DHK) (Keykha *et al*., 2016; Nakamura *et al*., 2018). As is shown in Fig. 1, DHK can be further hydroxylated at the 3' position or at both the 3′ and 5′, positions to produce dihydroquercetin and dihydromyricetin, respectively. DHK can also produce brick-red/orang pelargonidin. red/pink cyanidin-, and blue/violet delphinidin-based pigments can also be produced from dihydroquercetin, and dihydromyricetin respectively which shows the necessity and importance of these three biosynthesis pathways for diverse flower colors (Freyre *et al*., 2015; Azadi *et al*., 2016).

Current research showed higher *chi* gene expression (key enzyme in flavonoids biosynthesis pathway and color production) of red flowers in comparison with other colors. Results construct two hypotheses. First, red pigment production pathway may be the premier pathway in this plant. So, the red pigment production pathway can be assumed as the basic pathway in anthocyanins production. Probably, other pathways (pink and blue pigments production pathways) derive from

this pathway with the involvement of certain enzymes. Therefore, presumably upstream enzymes in this pathway (red pathway) such as chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone 3 hydroxylase (F3H) have high expression rather than other pathways, because can provide these enzymes for other pigment production such as pink and blue pathway.

Second, color production pathway in plants with different flower's color can be communal. In other words, total petunias with various colors have a common biosynthesis pathway for pigment production and only physiological and environmental conditions activate flavonoid 3´ hydroxylase (F3´H) and flavonoid 3´, 5´ hydroxylase (F3´5´H) enzymes in certain time points that produce pink and blue pigments, respectively (Brugliera *et al*., 2013; Kallam *et al*., 2017). For example, an environmental factor influence anthocyanins biosynthesis pathway is vacuolar pH. So that, pH variation is effective on pigment production. Studies had indicated in most plant species that acidic pH lead to produce blue, purple and violet colors and alkaline pH had played a significant role in red pigment production. However increasing in flavonoids and chi expression in all petunia flowers rather than white flowers seems to be the reason for high activity and expression of *chi* in other colors. In this regard, it is reported that presence of this gene is necessary for pathway continuity and pigments production (Hsu *et al*., 2017; Fujino *et al*., 2018). In other words, *chi* gene is the second gene in anthocyanins production pathway. If primary genes don't exit, anthocyanins production cycle is ceased and the pigment would not be produced. But, *chi* expression reduction in fully-opened flowers stage can relate to improve gradually anthocyanins biosynthesis pathway. When flowering completes (flowers are fully-opened), pigments production is completed too and final flower color is appeared. In other words, in primary flowering process (flower's bud), because starting of anthocyanins cycle, *chi* gene has more activation and take pathway towards target anthocyanins. Hence, when plant arrive to final flowering process (fully-opened flowers), activation of this gene is reduced.

Measuring concentrations of flavonoids and anthocyanins showed (regardless of flower's color) that the maximum concentrations is in final stages when flowers are fully-opened (Table 3). In the final stage of flowering, color production is completed in petals and flowers show their final color. It is expected that with the completion of color biosynthesis cycle, the quantity of total anthocyanins in petals increase. Since, the anthocyanins are the part of flavonoids biosynthesis pathway, with enhancement of anthocyanins, the quantity of total flavonoids increases in petals. Results of this research confirm this theory.

Obtained results in this experiment are corresponding with published reports. Koseki *et al*., (2005) were investigated 5 gene expression in pigment production (chalcone synthase, chalcone isomerase, flavanone 3 hydroxylase, dihydrokaempferol 4 reductase and anthocyanidin synthase) in petunia flowers. In above study, the expression of all studied genes were increased in red petals in compare to white petals.Griesbach *et al*., (2007) were investigated of chalcone synthase gene expression. In their study, the most expression was reported in red petals. Also, peach plants showed significant different between chalcone synthase (*chs*) and chalcone isomerase (*chi*) gene expression in red and white flowers. So, red petals have the most expression of chs. *Chi* gene showed increasing expression in red petals in comparison of white flowers, but the difference was not significant (Chen *et al*., 2014).

Generally, final concentration of anthocyanin in plant cells are not solely determined by expression levels of structural gene, and it is believed that some regulatory genes, especially specific transcription factors, should be involved in controlling pattern and intensity of anthocyanin biosynthesis. The bHLH, MYB and WD40 are three classes of reported TFs related to flavonoid biosynthesis (Sun *et al*., 2015). However, their importance in different species has not been approved or reported up to now. Our results introduce some genes associated with petal color variegation in

studied petunia flowers which could be extended using reverse genetic studies such as RNAi and CRISPER techniques. These methods-based studies may facilitate providing unique insights into the molecular mechanisms controlling variated flower pigmentation, and may eventually lead to the molecular engineering of plants.

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