Research Article

Analysis of Chalcone Synthase and Chalcone Isomerase Gene Expression in Pigment Production Pathway at Different Flower Colors of *Petunia Hybrida*

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Received 06 October 2015 Accepted 10 November 2015

Abstract

Variegation in flower color is commonly observed in many plant species and also occurs on petunia (*Petunia hybrida*) as an ornamental plant. Variegated plants are highly valuable in the floricultural market. To gain a global perspective on genes differentially expressed in variegated petunia flowers, we investigated the expression of chalcone synthase (*chs*) and chalcone isomerase (*chi*) as two essential genes in biosynthesis pathway of pigment production. Also, we measured the concentration level of total flavonoids, naringenin chalcone and naringenin to evaluate the probably relationship between the expression profile of *chs* and *chi* genes and the concentration of mentioned pigments. The results indicated that chalcone synthase and chalcone isomerase expression had different profile in different petal color of *Petunia hybrida*. Because red flower color in petunia is related to the synthesis of pelargonidin-based (orange to red) pigments, our results suggest that the low chalcone synthase and chalcone isomerase expression levels in white petals reduce dihydrokaempferol formation, thereby inhibiting pelargonidin production. In contrast, the high expression levels of these genes observed in red petals ensure sufficient anthocyanin yields to make flowers red.

***Keywords*:** Real-time PCR, *Chs*, *Chi*, *Petunia hybrida*

Introduction

Flower color is one of the most important characteristics in ornamental plant breeding. Flavonoids are the best characterized plant-specific secondary metabolites that accumulate in a broad range of plants, from gymnosperms to angiosperms (He et al., 2013). Flavonoids are natural products that contain a C6-C3-C6 carbon framework. They have a wide variety of biological functions including protection of cells against UV radiation, defense against pathogens and herbivores, regulation of auxin transport, and signaling between plant-microbe interactions, pollen growth and development, root nodule organogenesis, and most importantly, the flower colors facilitate attraction of pollinators and disperse the fruits and seeds. They are largely responsible for diverse pigmentation in the flowers, fruits, seeds, and leaves from shiny orange to pink, red, violet and blue (Kanazawa et al., 2007; Tan et al., 2013).

Flavonoids, a class of low-weight phenolic compounds, are derived from the general phenylpropanoids pathway. So far, more than 9,000 flavonoids have been identified. They are one of the most important secondary metabolites that 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 (Liu et al., 2010; Tan et al., 2013).

Despite of the complexity of the flavonoid biosynthetic pathway, flower coloration is specifically connected to the flavonoid biosynthetic pathway (Liu et al., 2013; Tan et al., 2013). 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, the gene encoding the chalcone synthase enzyme (CHS) is the first dedicated one in this pathway, which catalyzes the stepwise condensation of three molecules of malonyl-CoA to one molecule of 4-coumaroyl-CoA to naringenin chalcone get synthesized, leading phenylpropanoids pathway to flavonoids biosynthesis (Figure 1). Production of chalcone starts with the transfer of a

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coumaroyl moiety from a p coumaroyl-CoA starter molecule to an active site cysteine (Cys164). Then, a series of condensation reactions of three acetate units derived from three malonyl-CoA molecules, each proceeding through an acetyl-CoA carbanion derived from malonyl-CoA decarboxylation, extends the polyketide intermediate. Following generation of the thioester-linked tetraketide, a regiospecific intramolecular claisen condensation forms a new ring system to yield chalcone (Yang et al., 2003; Dao et al., 2011).

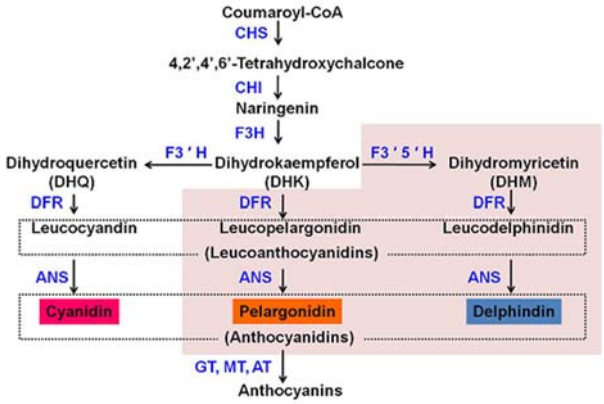

Figure 1. The flavonoid biosynthetic pathway leading to the synthesis of pigments (Adopted from He et al, 2013).

The CHS enzyme is also known as a type III of polyketide synthase enzymes (PKS) that is structurally and mechanistically the simplest PKS. These enzymes operate as homodimeric iterative PKS (monomer size of 42–45 kDa) with two independent active sites that catalyze a series of decarboxylation, condensation, and cyclization reactions (Deng et al., 2014).

The second key enzyme in flavonoid biosynthesis pathway is chalcone isomerase (CHI). This enzyme catalyzes the isomerization of naringenin chalcone into the corresponding flavanone (Figure 1) (Tunen et al., 1988). 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 attracted much attention recently because of its involvement in the stress response and pigment production (Weely et al., 1983; Tunen et al., 1987). Petunias (*P. hybrida*) are one of the best annuals flowering plants for mass display in gardens and streets, and they also can be used for borders, containers, hanging baskets or as seasonal ground cover. They have a long flowering period, which can be from spring until frost occurs. Their flowers color range is large. It is much-loved, widely grown worldwide and plays an important role in improving the city environment (Wang et al., 2006). Understanding mechanisms lead to pigmentation of flowers is the first step for manipulation the flower color that is desirable especially in ornamental plants.

To investigate and evaluate the probably relationship between the expression profile of *chs* and *chi* genes and the concentration of total flavonoids, naringenin chalcone (4,2',4',6'-Tetrahydroxychalcone) and naringenin, four distinct color (red, blue, pink and white) of *P. hybrida* were selected and assessed in present study.

Materials and Methods

Plant material

Potted plants of *P. hybrida* were grown under standard greenhouse conditions (16-17°C night temperature and 21-24°C day temperature and photoperiod 16/8 (light/dark)). Expanded white, pink, red and blue petals of *P. hybrida* were separately collected (Figure 2) and immediately immersed in liquid nitrogen after excision and preserved in a -80°C ultra-low temperature freezer until RNA extraction. Simultaneously, the same petal tissues were gathered to measure the mentioned pigments content.

Figure 2. The commercial varieties of *Petunia hybrida* with different color of petals. From left to right: red, blue, pink and white petunia.

RNA extraction and cDNA synthesis

Total RNA was extracted separately from four colors of petunia (white, pink, red and blue) 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 determined 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 *chs* and *chi* genes were designed to amplify 188 and 137 bp fragments, respectively (Table1).

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Table 1. Sequences of primers used for the amplification of *chs*, *chi* and *ef1A* genes in *P. hybrida*.

Primers/Genes	chalcone synthase (*chs*)	chalcone isomerase (*chi*)	elongation factor (*ef1A*)
Forward primer (5'→3')	AGACATAGTGGTGGTTGAAGTG	TCTCCTCCAGTGTCCGTTAC	TAAGTCTGTTGAGATGCACC
Reverse primer (5'→3')	TGAGCCTCTTGACCGATGG	ACAAACTTCCCTTCTATCTCCAG	CTGGCCAGGGTGGTTCATGAT

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/10min followed by 95°C/15sec, 60°C/15sec and 72ºC/30sec (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.

Statistical Analysis

As mentioned above, our 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 statistically analyzes of the data were done using T-student and Tukey's range test ($\alpha = 0.05$; JMP v8).

Results

Gene expression profile in different flowers of *P. hybrida*

The expression profile of two genes, chalcone synthase (*chs*) and chalcone isomerase (*chi*) were investigated in four *P. hybrida* colors (white, blue, pink, and red) when petals were completely opened. At this stage, the PCR products of all primers were coincided with bioinformatically predicted lengths.

The highest level of *chs* expression was observed in red flowers petals with 4.6 times more than white flowers. In blue and pink flowers, the expression of *chs* gene had increased up to 4.1 and 3.3 times, in compare to the white color ones although there was not a statistically significant difference between these two colors together (Figure 3).

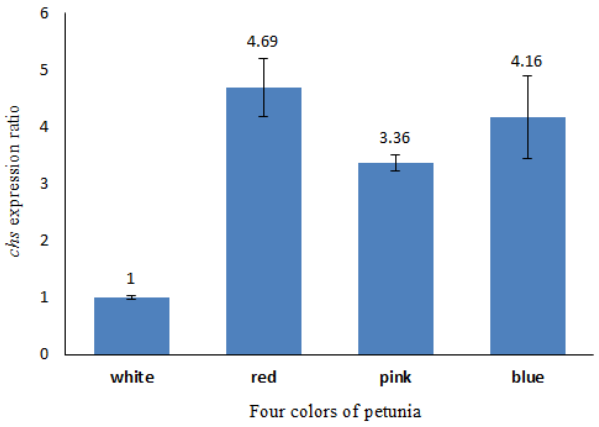

Figure 3. *Chs* gene expression in four colors of *P. hybrida*. Each data represents the average of three independent experiments. Error bars indicate the standard errors of the average of *chs* expression.

The analysis of *chi* expression in four *P. hybrida* also showed a significant expression increase of this gene in, red, blue and pink petals, compared to plants with white as control ones. *Chi* gene expression showed 3.27 times increase in plants with red flowers and 2.69 and 2.4 times increase in blue and pink flowers, relatively. However differences between blue and pink flowers were not statistically significant (Figure 4).

Measurement of total flavonoids, naringenin chalcone and naringenin

As was mentioned above, optical absorbance in 415 nm wavelength shows the level of total flavonoids in petal extract. Our results showed that

the absorbance of red, blue, pink, and white petals at this wavelength were 9.57, 5.95, 1.86, and 0.34, respectively (Figure 5).

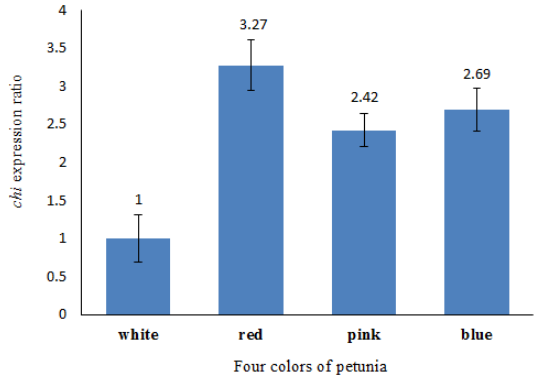

Figure 4. *Chi* gene expression in four colors of *P. hybrida*. Each data represents the average of three independent experiments. Error bars indicate the standard errors of the average of the *chi* expression.

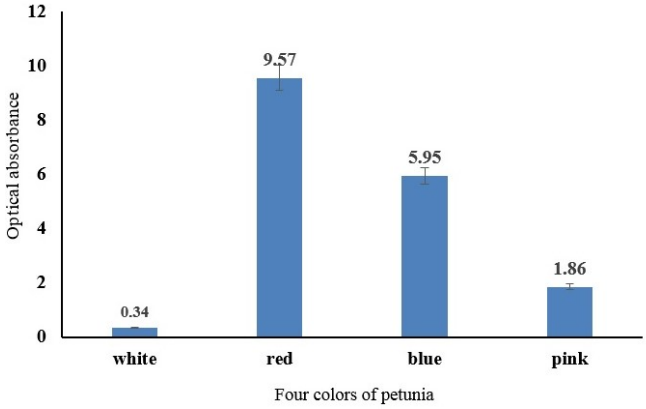

Figure 5. Optical absorbance of four *Petunia hybrida* in 415 nm that shows the level of total flavonoids. Each data represents the average of four independent experiments. Error bars indicate the standard errors of the average of the total flavonoid content.

Optical absorbance at 369 nm (related to naringenin chalcone concentration) showed an increase in petals with red, blue, and pink color in comparison with white ones. The highest optical absorbance at 369 nm was observed in red flowers (45.6), with 3.5 times more than white ones. The absorbance at 369 nm of blue and pink flowers was 30.3 and 15.2, respectively, however, the differences of pink and white petals were not statistically significant (Figure 6).

In order to estimate the relative concentration of naringenin,optical absorbance at 290 nm wavelength were measured in all four colors of petunias. Optical absorbances of red, blue and pink flowers were about 2.5, 1.6 and 1.07 times more than white flowers, while pink and white petal flowers showed statistically equal absorbance at 290 nm (Figure 7).

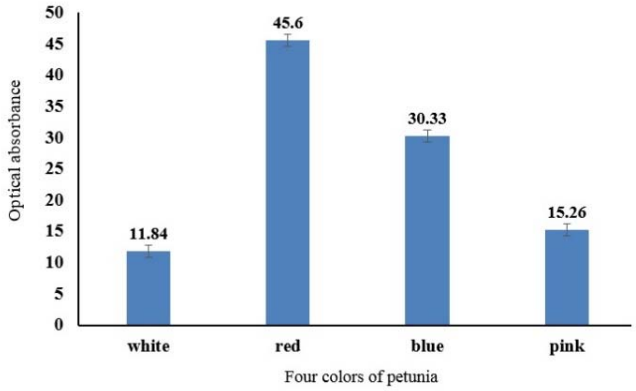

Figure 6. Optical absorbance of four *Petunia hybrida* in 369 nm that shows the level of naringenin chalcone. Each data represents the average of four independent experiments. Error bars indicate the standard errors of the average of the naringenin chalcone content.

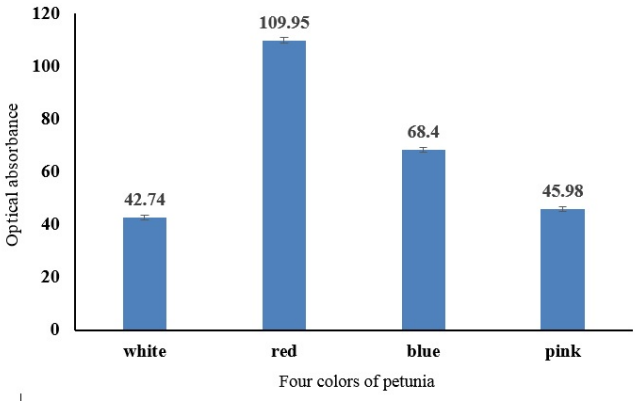

Figure 7. Optical absorbance of four *Petunia hybrida* in 290 nm that shows the level of naringenin. Each data represents the average of four independent experiments. Error bars indicate the standard errors of the average of the naringenin content.

Discussion

Flower color is largely determined by flavonoids pigments. Anthocyanins are a major colored class of flavonoids that are responsible for the pink, red, violet and blue colors of flowers and other tissues. Briefly, the anthocyanin biosynthesis pathway begins with the formation of chalcones by previously explained CHS. Then, CHI converts chalcone into naringenin. Naringenin is then hydroxylated at 3 positions of its central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK). DHK can then be further hydroxylated at the 3' position or at both the 3′ and 5′ positions of the B-ring to produce dihydroquercetin and dihydromyricetin, respectively (Figure 1).

DHK, dihydroquercetin, and dihydromyricetin generally result in the production of brick-red/orang

pelargonidin-, red/pink cyanidin-, and blue/violet delphinidin-based pigments, respectively. Thus, the establishment of these three biosynthesis pathways is essential for diverse flower colors (Tan et al., 2013; Ma et al., 2014).

In this study, expression profile of *chs* and *chi* genes were studied at the completely open petals stage of all samples. The reason for choosing this step of flowering is completion of biosynthesis cycle for pigment production in each flower and appearance of their specific petal color.

Results of our experiments showed an expression increase in both *chs* and *chi* genes in plants with extreme flower colored ones (red and blue) in compared to other plants. The increase in optical absorbance at 369nm (naringenin chalcone) in red flowers can be attributed to higher expression of *chs* and presence of CHS (Figure 6). However, there was not any significant difference between *chs* expression of red and blue petal plants and *chi* expression of pink and blue ones. The different absorbance levels show different naringenin concentration in each petal color that can be attributed to more activities of chalcone isomerase enzyme.

Red flowers not only indicated the highest expression ratio of *chs* and *chi,* but also showed the highest concentration of total flavonoids, naringenin chalcone and naringenin. This pattern was not observed in pink and blue petal plants.

The reason for this might be attributed to two hypotheses. One is that red pigment production pathway in this plant is the main one compared to other pigments. As mentioned earlier and is shown in Figure 1, the pathway for red pigment production is the main pathway in anthocyanin production cycle, and other pathways that lead to blue and pink pigments are somehow derivation of this pathway by intermediate special enzymes. Therefore, upstream enzymes of this pathway (the pathway for red pigment production) like CHS, CHI and F3H must be more than other pathways to somehow provide these enzymes for other pigment production pathways like white and pink pigments.

The second hypothesis can be the common pathway for pigment production in plants with different flower colors. In other words, all petunia flowers with different colors have a common biosynthesis pathway to produce pigments, and only various physiological and environmental conditions cause activation of two flavonoid 3′ hydroxylase (F3′H) and flavonoid 3′, 5′ hydroxylase (F3′5′H) enzymes during a certain time period that its outcome is production of blue and pink pigments. For example, one of the environmental factors that affects anthocyanin biosynthesis pathway is pH of vacuole environment in a way that variations in pH, explicitly affect pigment production and special color. Research shows that in most plant species, acidic pH causes purple, violet and blue colors (Vlaming et al., 1983).

Regarding the concentration of these two compounds in red flowers, but not other colors, ecologic role of red color in absorbance of pollinators, can also be pointed out, since most insects and birds are attracted toward flowers with red and orange colors. In other words, ecologic and evolutionary evidences show the role of natural selection in development of most flowers with red, orange, and yellow colors that will result in appropriate interaction of these flowers with insects and birds (Rodríguez-Gironés & Santamaría, 2004; Rodríguez-Gironés & Santamaría, 2010). Therefore, more activities of these two enzymes in red flowers can be attributed to selection of these flowers in an evolutionary process, which leads to more activity of effective enzymes in anthocyanin production, especially enzymes in red pigment production.

According to the results of naringenin chalcone and naringenin level measurements, it can be concluded that the main pathway and other pigments such as blue and pink were derived from red pigment biosynthesis pathway.

These results coincide with reports of Koseki et al. (2005) that studied five main genes in petunia red star flowers and showed that expression of genes in red petals were increased compared to white petals. Griesbach et al. (2007) also compared white and red star petunia plants and observed that highest expression of *chs* gene was in red petals. In the study of *chs* and *chi* gene expression from white and red flowers of peach, a significant difference was observed between the two colors as red petals devoted the most *chs* gene expression to themselves. According to the conducted studies, it seems that CHS and CHI are two main enzymes in flavonoids/anthocyanins biosynthesis pathway and finally pigment production pathway; in a way that without the presence of these enzymes, biosynthesis cycle is not complete and consequently no pigment would be produced (Napoli et al., 1990; Mori et al., 2004).

However, these two enzymes are not ultimate enzymes and determiner of pigment type in plants with different flower color. In other words, although the presence of these two enzymes is essential for the initiation of pigment biosynthesis cycle in all studied plants, but their presence and concentration are not reliable indexes for predicting the final color of plant flowers.

As mentioned above, F3′H, F3′5′H and enzyme concentration of dihydroflavonol 4-reductase (DFR) can be pointed out as the main and effective enzymes in production of different colors resulted from anthocyanins biosynthesis pathway that produces pink, blue and red pigments, respectively (Pang et al., 2005; Sun et al., 2015).

Generally, final anthocyanin concentrations in plant cells are not determined solely by structural gene expression levels, and it is expected that some regulatory genes are obviously involved in the control of flavonoid biosynthesis. These regulatory genes, especially specific transcription factors, influence expression of many different structural genes that generally control pattern and intensity of anthocyanin biosynthesis. Up to now, three classes of TFs (bHLH, MYB and WD40) have been reported that seems to be related to flavonoid biosynthesis (Sun et al., 2015), however, further studies are needed to approve their importance in different variation. Our results uncover candidate genes associated with variegation in studied variety of petunia flowers. We also found that the higher expression of both *chi* and *chs* leads to higher concentration of studied pigments but the proportion of each one separately is not clear as was observed in blue and pink colored petals with different expression ratio of *chs* and *chi* in contrast to white and red colored flowers. Using reverse genetic may facilitate resolving this problem and providing unique insights into the molecular mechanisms controlling variegated flower pigmentation, and may eventually help the molecular engineering of variegated plants.

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