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اثر پرتودهی گاما بر بیان ژن‌های ساختاری دخیل در مسیر ساخت آنتوسیانین در گل داوودی

Effect of gamma irradiation on the expression of structural genes involved in anthocyanin biosynthesis pathway in chrysanthemum

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چکیده

گل داوودی (*Chrysanthemum morifolium*) **یکی از مهم‌ترین گیاهان زینتی ایران و جهان است که دارای ارزش تجاری بالایی می‌باشد. با بررسی دزهای مختلف پرتو گاما، پرتوتابی با دز بهینه ۴۰ گری توسط چشمه کبالت ۶۰ به گیاهچه‌های چهار هفته‌ای یک رقم تجاری گل داوودی به نام Rambla به رنگ بنفش، باعث ایجاد سه جهش‌یافته با رنگ‌های بنفش پررنگ، بنفش کم‌رنگ و جگری گردید. به منظور مطالعه اثر تغییرات سطح بیان ژن‌های ساختاری دخیل در مسیر تولید آنتوسیانین بر رنگ جهش‌یافته‌ها، RNA نمونه‌های جهش‌یافته و نمونه‌های شاهد از گلبرگ‌های نسل M1V3 این گل‌ها استخراج شد و توسط روش RT-qPCR مورد بررسی قرار گرفت. تغییر معنی‌دار سطح نسبی بیان ژن‌های *F3'H*، *DFR*، *ANS* و *3GT* در جهش‌یافته‌ها نسبت به نمونه شاهد، بیانگر نقش این ژن‌ها در تغییر رنگ جهش‌یافته‌ها نسبت به نمونه شاهد بود. همبستگی محتوای آنتوسیانین کل با سطح نسبی بیان ژن‌های *DFR*، *ANS* و *3GT* معنی‌دار بود. رگرسیون گام به گام برای محتوای آنتوسیانین کل به عنوان متغیر وابسته و سطح نسبی بیان ژن‌های ساختاری دخیل در مسیر تولید آنتوسیانین به عنوان متغیرهای مستقل نشان داد که ژن *3GT* بیشترین تاثیر را بر متغیر وابسته داشت. ژن *F3'H*، که بیان نسبی آن در جهش‌یافته‌ها نسبت به نمونه شاهد تغییر معنی‌دار نشان داد، به عنوان ژن کلیدی مناسب جهت دست‌ورزی ژنتیکی به منظور ایجاد تنوع رنگ در گل داوودی معرفی می‌گردد.**

واژه‌های کلیدی

آنتوسیانین،
پرتو گاما،
جهش‌یافته،
ژن‌های ساختاری،
گل داوودی

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Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat) belongs to the family Asteraceae and is grown by root suckers and terminal cuttings (Kalia 2015). Chrysanthemum is one of the most important ornamental plants used as not only a cut flower but also a garden, potted, and medicinal plant. It has two general types including standard (cut flower) and spray (Siddiqua *et al.* 2017). Chrysanthemums are short-day (Teixeira da silva *et al.* 2013) and heterozygous (Fukai *et al.* 2000) with nine basic chromosomes (x=9). Chrysanthemums have different ploidy levels from diploid to decaploid, but its commercial species are hexaploid (2n = 6x = 54) (Liu *et al.* 2012; Chatterjee *et al.* 2005). Its genome length has been estimated at 9.4 Gb (http://data.kew.org/cvalues/). Most breeding programs aim to improve the ornamental features, color, size, and shape of the flowers (Teixeira da silva 2004). As long as ornamental species are concerned, flower color is a decisive factor of quality that affects not only the ornamental value of the plant but also its commercial value (Zhao and Tao 2015). It is of high significance to create genetic potentials and evaluate the major commercial traits for the sake of introducing new cultivars of ornamental plants (Azimi 2020a,b; Azimi and Karimi Alavijeh 2020). Genomic complexity, aneuploidy, high levels of heterozygosity, and sporophytic self-incompatibility of chrysanthemums are the main barriers against the production of pure lines and are responsible for the low genomic information we have about this plant species (Xu *et al.* 2013; Zhang *et al.* 2013). Also, the genetic improvement of chrysanthemums with classic methods is time-consuming and difficult (Kishi-Kaboshi *et al.* 2017). Anthocyanin production imparts diverse colors to chrysanthemums, which improves their ornamental value (Liu *et al.* 2016). Due to the low frequency of spontaneous mutations for diversification, researchers use mutation induction techniques as a useful tool for creating or increasing the diversity of plant species (Novak and Brunner 2000). The induced mutation is a rich source for the diversity required by chrysanthemum genetic improvement programs (Momin *et al.* 2012). When the mutation occurs in the genes responsible for the anthocyanin biosynthesis pathway, the accumulation of certain anthocyanin results in changes in the flower color (Mato *et al.* 2000; Hongmei *et al.* 2009). Research in which chrysanthemums have been exposed to gamma irradiation has reported the creation of diverse colors in this plant. For example, the gamma irradiation of pink-color chrysanthemum ‘Argus’ seedlings created three mutants – two in purple at the dose of 40 Gy and one in white at the dose of 50 Gy (Park *et al.* 2007). The 2 kilorad gamma irradiation of dark yellow chrysanthemum ‘Akola Local’ seedlings resulted in the production of a brick-red mutant (Momin *et al.* 2012). When pink chrysanthemum ‘Otome Pink’ seedlings were exposed to gamma irradiation at the doses of 10, 15, and 20 Gy, yellow, orange, and light pink mutants were obtained at the doses of 10 and 15 Gy (Kumari *et al.* 2013). The gamma irradiation of four chrysanthemum varieties, including CN01, CN20, CN93, and CN93, at the doses of 5, 10, 20, and 30 Gy revealed petal color change at the doses of 20 and 30 Gy (Thao *et al.* 2015). The seedlings of two chrysanthemum cultivars, including ‘Noble Wine’ and ‘Pinky’, exposed to 30-Gy gamma irradiation produced a range of colors in their mutants (Kim *et al.* 2016). A study on 23 chrysanthemum cultivars focused on the relationship between total anthocyanin content and the color intensity of the petals and revealed that the purple and red flowers had the highest total anthocyanin content (Park *et al.* 2015). The exposure of pink chrysanthemum ‘Argus’ seedlings to 30, 40, and 50 Gy gamma irradiation resulted in the production of white and purple mutants. The white mutant had four times lower total anthocyanin content and the purple mutant had about six times higher total anthocyanin content than the control cultivar. This study did not observe a relationship between the expression of the genes *CHS* and *F3H* at the upstream of the anthocyanin biosynthesis pathway and the flower color. The researchers suggest that mutation at downstream of the anthocyanin biosynthesis pathway can make changes in flower color (Lee *et al.* 2008). There is a report as to the relationship between the expression of the genes *DFR* and *3GT* in downstream of the anthocyanin biosynthesis pathway with anthocyanin content and petal color intensity of chrysanthemums (Chen *et al.* 2012).

Flavonoids are important plant pigments that are responsible for flower pigmentation (Veitch and Grayer 2008). These pigments are synthesized through the phenylpropanoid pathway from the amino acid phenylalanine (Falcone Ferreyra *et al.* 2012). Anthocyanidins (Fig. 1) are the building blocks of anthocyanins (Castaneda-Ovando et al. 2009). They belong to the group of flavonoids and play a key role in flower pigmentation (Tanaka *et al.* 2008).

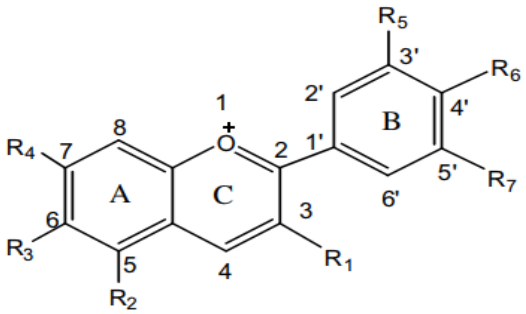

Fig 1. General anthocyanin structure (Castaneda-Ovando et al. 2009)

There are three primary types of anthocyanidins – cyanidin, delphinidin, and pelargonidin. These anthocyanidins differ in the number and nature of hydroxyl groups, the number and replacement of sugar molecules (attached to 3'C and 5'C) in their structures, the

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type of carboxylates attached to the sugar molecule, and the location of these attachments (Kong *et al.* 2003). The anthocyanin biosynthesis pathway is a branch of the flavonoid biosynthesis pathway (Fig. 2). The structural genes involved in anthocyanin biosynthesis including the upstream genes of *CHS*, *CHI*, *F3H*, *F3'H*, and *F3'5'H* and the downstream genes of *DFR*, *ANS*, and *3GT*, which produce the enzymes chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3'-hydroxylase, flavonoid 3'5'-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase, and 3-*O*-glucosyltransferase, respectively, thereby playing a role in the pigmentation of chrysanthemum (He *et al.* 2013).

The sequence of the genes involved in the anthocyanin biosynthesis pathway is severely conserved in different plant species (Zhang *et al.* 2013). Phenylalanine is converted to coumarate-CoA by making some chemical modification with the aid of the enzymes phenylalanine ammonia-lyase, cinnamate-4-hydroxylase, and 4-coumarate CoA ligase. Then, one molecule of coumarate-CoA and three molecules of malonyl-CoA form coumaryol-CoA (Zhao and Tao 2015). Then, it is converted to chalcone by the CHS enzyme. The CHI enzyme converts chalcone into naringenin, which is a flavonoid. Next, naringenin is hydroxylated by the F3H enzyme to turn into DHK, which is necessary for color diversity. At the next step, DHK can be hydroxylated by the F3'H and F3'5'H enzymes in 3'C locus and/or in both 3'C and 5'C loci of the anthocyanin B-ring, respectively for the biosynthesis of DHQ and DHM. F3'H competes with F3'5'H in the anthocyanin biosynthesis pathway (He *et al.* 2013) but cooperates with CHS and CHI to accelerate the downstream production in the anthocyanin biosynthesis pathway (Owens *et al.* 2008).

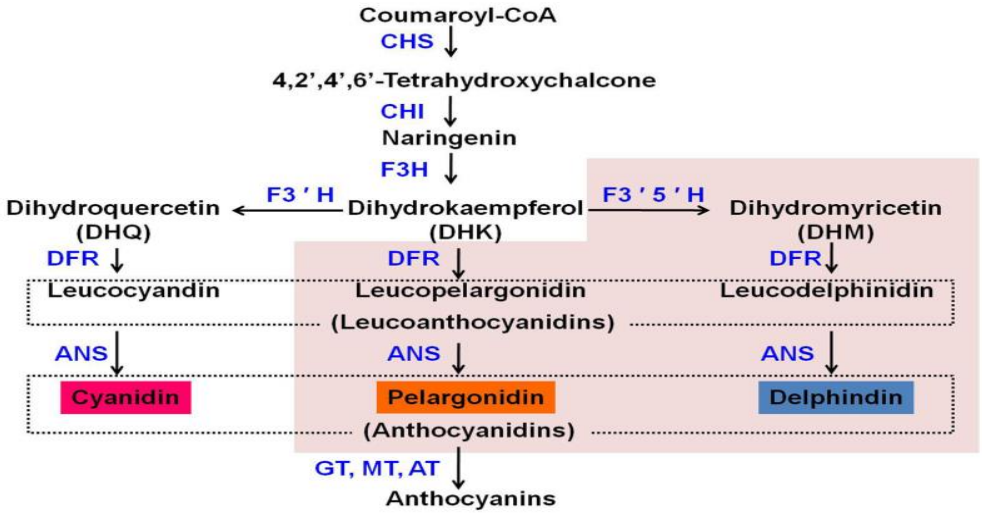

Fig 2. The anthocyanins biosynthetic pathway (He et al. 2013)

Then, DFR, which is a key enzyme in the anthocyanin biosynthesis pathway, converts DHK, DHQ, and DHM into leucopelargonidin, leucocyanidin, and leucodelphinidin, respectively. After that, anthocyanidin synthase changes these structures into the anthocyanidins of pelargonidin, cyanidin, and delphinidin by (He *et al.* 2013; Zhao and Tao 2015) and is involved in their accumulation in chrysanthemum (Lee *et al.* 2008). The inactivity of the gene *ANS* would cause the reduction of anthocyanin accumulation in flower petals (Rosati *et al.* 1999). The 3GT enzyme, which glycolyzes anthocyanidins to anthocyanins (Chen *et al.* 2012) and causes their pigmentation, is not only important for the synthesis of pigments but also necessary for the stability of the structure of anthocyanidins so that they can be accumulated in plants as water-soluble pigments (Schijlen *et al.* 2004). The anthocyanins pelargonidin, cyanidin, and delphinidin are responsible for the colors orange, red, and blue in ornamental plants, respectively (Falcone Ferreyra *et al.* 2012). The knowledge of how the structural genes involved in the anthocyanin biosynthesis pathway of chrysanthemums are expressed can contribute to understanding the molecular mechanism of color formation by anthocyanins (Hong and Dai 2015). The present study aimed to explore the variation in the relative level of the expression of structural genes involved in the anthocyanin biosynthesis pathway in cut chrysanthemums exposed to gamma irradiation.

MATERIALS AND METHODS

Chrysanthemum morifolium cv. 'Rambla', which is purple and of the standard and hexaploid type, is a cultivar of cut chrysanthemum that is very popular in Iran. The rooted and healthy cuttings of this cultivar were treated with

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gamma irradiation at different doses (10, 20, 40, 60, and 80 Gy) to find out the optimal dose. The irradiation was performed in the Nuclear Science and Technology Research Center of Karaj with a cobalt-60 source at the optimal dose of 40 Gy irradiated to four-week-old seedlings that had been produced in May 2017. The result was the production of three mutants with petals in dark purple (M1), pale purple (M2), Indian red (M3) (Fig. 3).



Fig 3. C(control): purple and mutants M1: dark purple, M2: pale purple, M3: Indian red.

At the full flowering stage (October 2019), the petals of the M1V3 generation of the mutant plants and those of the control (non-irradiated) plants that were cultivated in research field conditions at Ornamental Plants Research Center of Mahallat, Iran (Lat. 33º54'30.13" N., Long. 50º27'30.34" E., Elevation of 1747 m.) were sampled, packaged separately, and immediately placed in liquid nitrogen tanks. The samples were then kept at -80ºC freezer until RNA extraction.

RNA extraction and cDNA synthesis: The total RNA of the petals was extracted by the p-BIOZOL buffer (Bioflux, Japan) according to the manufacturer's guidelines. The enzyme *DNase I* (Fermentas) was employed to eliminate the DNA that remained in the RNA samples. The quality and quantity of the extracted RNA were examined by the electrophoresis of the agar gel and spectrometry. The reverse transcription reaction was performed by the enzyme Mmulv (Fermentas) as per the manufacturer's guidelines. To normalize the error, the cDNA of the three control samples was mixed in equal ratios and it was used as the control cDNA. The quality of the cDNA produced was confirmed by the housekeeping gene primer *ACTIN* with the standard PCR.

Primer design: The sequence of the transcription of the structural genes involved in the anthocyanin biosynthesis pathway of chrysanthemum was derived from the NCBI website. Specific primers to amplify the transcription of the genes were designed in Primer 3 software (Table 1).

Table 1. The specifications and sequence of the primers used in the study

Gene	ID	Sequence	Tm(°c)	PCR product size	References
ACTIN	AB205087	F: ACTACAACTGCTGAACGGGA R: AATCGTGATCACCTGCCCA	56.8	150	Puangkrit et al. 2018
CHS	DQ521272	F: GGAAGAAGTCGGCTGAGGA R: TAGTGGTTGGGAGGCTGTG	57.8	123	He et al. 2013
CHI	EU286277	F: AGGACGAAAACTTCCCACCA R: CACCATTCTTCCCGATCACC	57.3	171	He et al. 2013
F3H	DQ471438	F: ACCTGACCTCACTTTGGGC R: ACCGTGATCCAAGTTTTGCC	58.1	120	He et al. 2013
F3'H	KF313549	F: GATTTGTGCGGGTATGAGCC R: CGCTTCTTCCATGTTGAGCA	57.1	124	He et al. 2013
F3'5'H	AAX19888.1	F: GAYATGGTKGTDGAGYTVATGAC R: TYCCARTCAAADGMDTGMAYCAA	54	-	Lee et al. 2008
DFR	GU324979	F: AAAGGCGGACACTCACGAA R: ACATTCCCAGTCCCGCAAT	58.3	140	He et al. 2013
ANS	EU810810	F: GGTTAGGATTTCTTGGGCGG R: TGATGAAAGGTTCGTGGCG	57.3	120	He et al. 2013
3GT	JF433952	F: ATTGGCGTATACTGCTGGTG R: CGAACCAGCCAATCATACCC	62	121	He et al. 2013

Since no specific primer has ever been designed for the gene *F3'5'H* in chrysanthemum, a degenerate primer was employed for the amplification of its transcription.

RT-qPCR: The quantitative RT-PCR was performed to amplify the transcriptions of the genes involved in the anthocyanin biosynthesis pathway using specific primers by the quantification method with an iCycler (BioRad). The qRT-PCR reaction solution was prepared at a volume of 20 µL in specific tubes. The solution contained 3 µL of cDNA (at a dilution of 1:20), 0.5 µL of each primer (10 µM), 9.9 µL of SYBR™ Green qRT-PCR master mix 2x, 0.5 µL of DMSO, and 5 units/µL of the enzyme *Taq* DNA polymerase and its volume was adjusted at 20 µL using twice-distilled water. The thermal cycles of the reaction

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included one cycle at 95°C for 3 minutes and 35 cycles at 95°C for 10 seconds for denaturation and the temperature was adjusted for the attachment of primers for 20 seconds and at 72°C for 20 seconds for amplification with three replications for each gene. *ACTIN* was used as an internal control gene for calculating the relative level of the expression of the studied genes. Optimal conditions were provided for the concentration of primers and the temperature of the primer attachment stage, which is a major factor in appropriate PCR reaction so that no non-specific product was produced during the reaction as confirmed by the melting curve (the observation of a single melting peak for each sample).

Antocyanin extraction: To extract total anthocyanin content, 1 g of the petal tissue was ground with 10 mL of acidic methanol (99% methanol + 1% hydrochloric acid) with three replications for each sample. The resulting extract was placed in darkness at 4°C for 24 hours. It was then centrifuged at 4000 g. Afterward, the supernatant was separated and its absorption was read at 550 nm with a Photonix Ar 2015 array spectrophotometer. The anthocyanin concentration was calculated by the equation

$$A = \varepsilon bc$$

in which A represents the absorption of the solution, ε represents the extinction coefficient (= 33000 cm^2/mol), b represents the cuvette width (= 1 cm), and c represents the anthocyanin concentration in mol/cm^3 (Wagner 1979).

Data analysis: The relative level of the expression of the studied genes versus the reference gene was analyzed by the REST software package (Pfaffl *et al.* 2002) and Pearson's correlation coefficient and stepwise regression were estimated by the SPSS software package.

RESULTS AND DISCUSSION

The results showed that the total anthocyanin content in the control and M1, M2, and M3 mutants was 0.0000322, 0.0000547, 0.0000134, 0.0000624 mol/cm^3, respectively (Fig. 4). The total anthocyanin content of the M1 and M3 mutants was 69.8% and 93.7% higher than that of the control, respectively, but it was decreased by 58.3% in the M2 mutant versus the control. Total anthocyanin content of all mutants had significant difference versus control ($p< 0.01$).

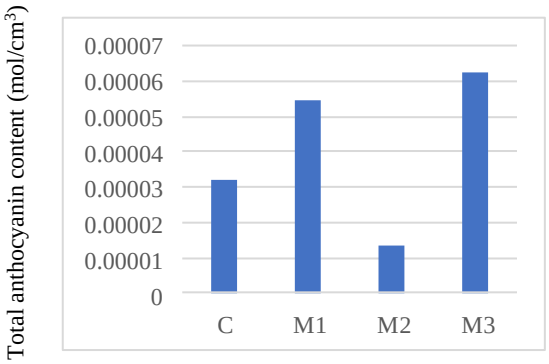

Fig 4. Total anthocyanin content in chrysanthemum petals of control (c) and mutants (M1, M2, and M3), Data shown are means of three replicates.

The results of the expression of the structural genes involved in the anthocyanin biosynthesis pathway revealed changes in the relative level of the expression of these genes (Fig. 5). The expression of *CHS*, *CHI*, and *F3H* was not significantly different in the mutants versus the control ($p> 0.05$). This implies that although the activity of the upstream genes of the anthocyanin biosynthesis pathway, such as *CHS*, *CHI*, and *F3H*, is necessary for the production of the substrate in this pathway, the changes in the expression of these genes were statistically insignificant, so they are unlikely to have played a key role in the color change of the studied mutants. Similarly, Lee et al (2008) reported the lack of a change in the pattern of the *CHS* and *F3H* expression in the mutants of gamma irradiation versus the control of chrysanthemum and did not find a relationship between the expression levels of these genes and the flower color. The suppression of the *CHS*, *CHI*, and *F3H* expression causes the petals of ornamental plants to become white (Schijlen *et al.* 2004; Zhao and Tao 2015). It was found that the mutants differed from the control significantly in the *F3'H* expression. The expression of this gene in the M1 and M3 mutants, which were darker than the control, was 10.5 and 4.1 times higher ($p< 0.001$).

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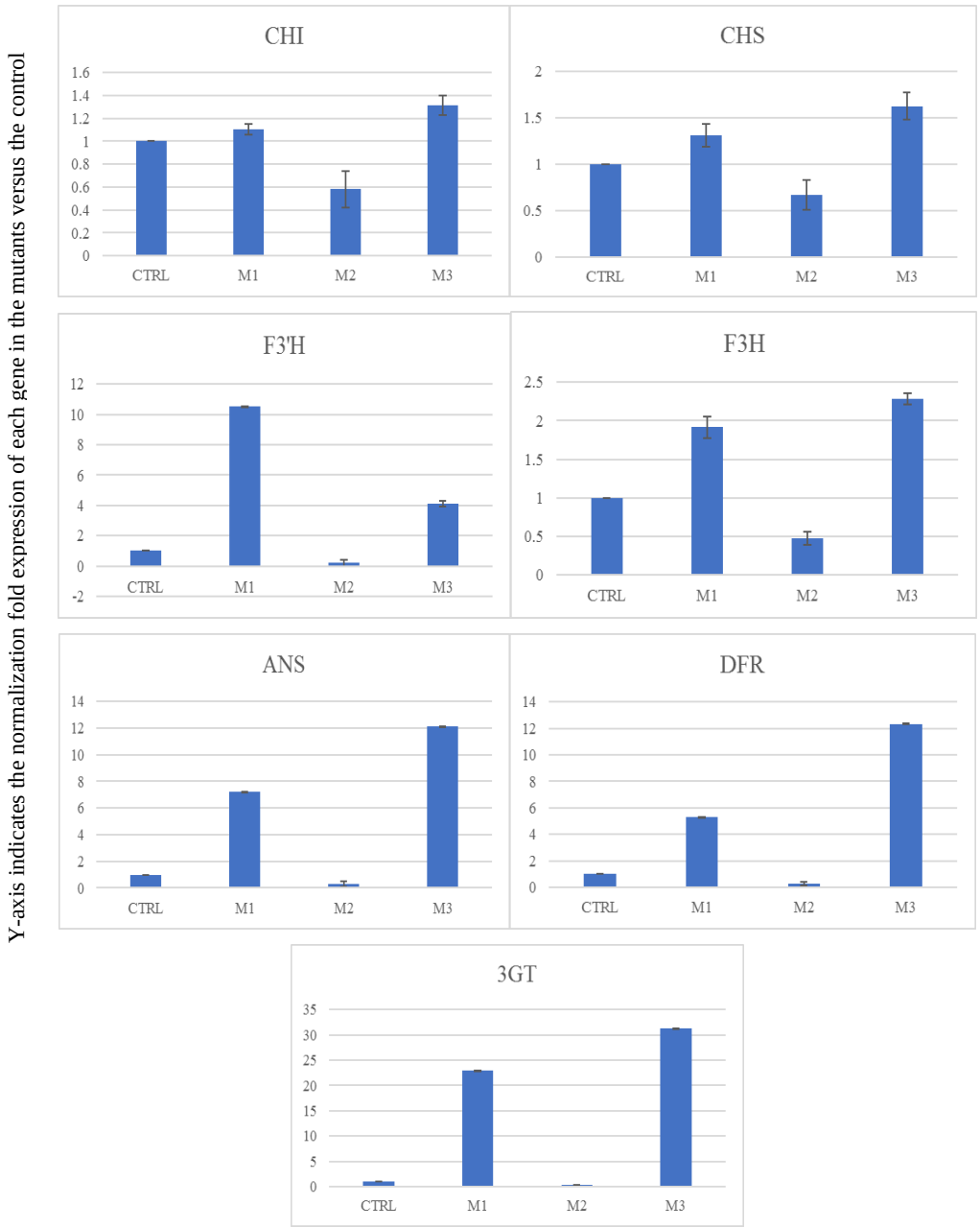

Fig 5. Relative expression of structural genes involved in anthocyanin biosynthesis pathway in chrysanthemum petals of control (c) and mutants (M1, M2, and M3), Data shown are means of three replicates, error bars denote standard error.

Also, this gene in the M2 mutant, which was paler than the control, exhibited a decrease of about 4.9 times in expression ($p< 0.001$). *F3'H* is responsible for the production of cyanidin and the amount of cyanidin in petals is the reason for the pink and red color of flowers (Ohmiya. 2018). It has been reported that the reduced expression of *F3'H* results in the reduction of the *DFR* expression (Zufall and Rausher 2004).

In the present study, the lighter color of the M2 mutant versus the control was likely to be related to the reduced expression of *F3'H* in the upstream of the anthocyanin biosynthesis pathway of this mutant, which reduced the substrate required for the activity of *DFR*, *ANS*, and *3GT* in the downstream of this pathway and limited cyanidin synthesis. Since the relative level of the *F3H* expression in the studied mutants did not change significantly ($p> 0.05$) versus the control, it seems that DHK produced by this gene either was not used in the pelargonidin synthesis pathway or the amount of pelargonidin synthesized was so slight that it could not produce orange color in the studied samples. The lack of orange in the studied samples shows

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that DHK was hydroxylated to DHQ and perhaps DHM by *F3'H* and *F3'5'H*, respectively. The fact that pelargonidin was not synthesized is associated with the over-activity of *F3'H*, which converts DHK to DHQ, finally resulting in the synthesis of cyanidin. Mutation by genetic engineering to suppress the *F3'H* expression can contribute to the synthesis and accumulation of pelargonidin in chrysanthemums (Ohmiya 2018). Some researchers argue that there is no blue chrysanthemums in nature because it lacks the gene *F3'5'H* (Brugliera *et al.* 2013; Kumari *et al.* 2019). But, others argue that this gene may have lost its performance over the course of chrysanthemum evolution and has gained its activity again by gene duplication (He *et al.* 2013). Although the degenerate primer could not observe the expression of *F3'5'H* in the present study, since it has been proven that the purple color of cineraria (Sun *et al.* 2009) and purple kiwifruits (Peng *et al.* 2019) is due to the concurrent occurrence of cyanidin and delphinidin in these plants and since the suppression of the *F3'5'H* expression in purple cyclamens and the lack of delphinidin synthesis change the color of this flower to red (Boase *et al.* 2010), F3'5'H was likely active in the studied mutants and the sample, so it has bestowed purple color to these flowers by synthesizing blue delphinidin and combining it with red cyanidin. The likelihood of the presence of *F3'5'H* can be checked by comparing the variations in the expression of *F3'H* in the M1 and M3 mutants as well as the total anthocyanin content. The facts that *F3'H* was expressed 2.5 times as great in the M1 mutant as in the M3 mutant but the total anthocyanin content of M3 was greater than that of M1 and that the M3 mutant had a darker color than the M1 mutant increases the likelihood of more activity of *F3'5'H* in the M3 mutant. It seems that in addition to the DHQ substrate (produced by the activity of *F3'H*), DHM (produced by the activity of *F3'5'H*) was also produced, so more substrate was supplied to the downstream genes of the anthocyanin biosynthesis pathway and this resulted in darker color of the M3 mutant than the M1 mutant.

A study reported that pink chrysanthemum 'Argus' produced white and purple mutants when exposed to gamma irradiation at 30, 40, and 50 Gy. The expression of *CHI*, *F3'H*, and *DFR* was increased in some mutants and decreased in others, whose effects were manifested in the color change of the petals. The extent of the *CHI* expression was increased in the purple mutant than in the control, but the extent of the *F3'H* expression was decreased. These researchers suggested that the presence of purple color among the mutants and the absence of red color were related to the common expression of *F3'H* and *F3'5'H* (Lee *et al.* 2008).

Different plant species usually produce specific types of anthocyanin, so limited colors are seen in plants (Tanaka and Ohmiya 2008). The use of genetic engineering to cope with this constraint (Tanaka *et al.* 2010) and diversification of the structural genes involved in the anthocyanin biosynthesis pathway are a major approach to diversifying flower colors (Noman *et al.* 2017). The flavonoid biosynthesis pathway of transgenic plants can be modified by gene transfer to the host plant and the use of the RNA interference technology to suppress gene expression in the host plant (Nakamura *et al.* 2006). As such, it is recommended to suppress the expression of *F3'H* by the RNA interference technology in both control and mutants studied here in order to study the presence or absence of *F3'5'H* expression considering the colors obtained in the petals by the suppression of this gene. Since the DFR enzyme has no substrate preference in chrysanthemums unlike other flowers like petunia and gerbera and can influence the structure of all three substrate types of DHK, DHQ, and DHM (Zhao and Tao 2015), in case of the activity of *F3'5'H* and the production of the DHM substrate, the DFR of the chrysanthemums will have no limitation in influencing this substrate and there will be no need to transfer the *DFR* gene to chrysanthemums from other flowers. Given the competition between *F3'H* and *F3'5'H* (He *et al.* 2013), after the *F3'H* expression is suppressed, if blue petals (even pale blue) is produced, it will be not only a success in producing blue chrysanthemums without gene transfer from other plants, but it will also confirm the presence and activity of *F3'5'H* in these samples.

The assessment of the expression of the genes involved in the anthocyanin biosynthesis pathway in the purple variety of chrysanthemum, i.e., 'H5', and two white cultivars including 'Keikai' and 'Jinba' showed the absence of the delphinidin and pelargonidin anthocyanins in these cultivars. The pattern of the expression of *F3H*, *F3'H*, *DFR*, and *3GT* in 'Jinba' was similar to that in 'Keikai', and all structural genes involved in the anthocyanin biosynthesis pathway were expressed both in the purple cultivar and in the white cultivars. The expression of the genes *DFR* and *3GT* were higher in cv. 'H5' than in white cultivars. These genes were introduced as the quantitative regulators, not the suppressors of the expression of other genes in the anthocyanin biosynthesis pathway (Chen *et al.* 2012). These researchers suggest that to understand whether *DFR* and *3GT* alleles bring about a color difference between white chrysanthemums and pink ones or their differential expression is caused by one or more regulating genes, further research is required. An approach to increasing delphinidin is to reduce the synthesis of cyanidin by reducing the activity of *F3'H* and to increase the synthesis of delphinidin by transferring *F3'5'H* and *DFR* from a plant like petunia to chrysanthemums because the DFR enzyme of petunia has the specificity for delphinidin synthesis (Tanaka *et al.* 2009). The increased expression of the *DFR* gene increases the anthocyanin content of petals and the difference in the expression of *DFR* and its substrate specificity create color variation in some flowers, studies of the mechanism of this gene on flower color development have become research priorities of researchers (Zhao and Tao 2015).

The relative level of gene expression in this research revealed that *DFR*, *ANS*, and *3GT* were expressed to a greater extent in the M1 and M3 mutants than in the control (these two mutants were darker than the control), but their expression was decreased in the M2 mutant

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compared to the control (the M2 mutant had paler color than the control). The *DFR* gene expression was 5.2 times ($p< 0.05$) higher in the M1 mutant than in the control and 12.3 times ($p< 0.05$) higher in the M3 mutant than in the control, but it was 3.4 times ($p< 0.05$) lower in the M2 mutant than in the control. The greatest increase in the *ANS* gene expression versus the control was related to the M3 mutant followed by the M1 mutant in the next rank. The *ANS* gene was expressed in the M1 and M3 mutants to a greater extent than in the control (7.1 and 12.1 times, respectively) ($p< 0.001$), whereas its expression was decreased in the M2 mutant by 3.3 times versus the control ($p< 0.05$). The expression of *3GT* was 22.9 and 31.2 times higher in the M1 and M3 mutants than in the control, respectively ($p< 0.001$), but 3.1 times lower in the M2 mutant than in the control ($p < 0.05$). Higher relative level of the expression of *DFR*, *ANS*, and *3GT* in the M3 mutant than in the M1 mutant and darker color of the former mutant than the latter one may be associated with more activity of the *F3'5'H* gene in the M3 mutant and consequently, higher content of the DHM substrate. The decline in the relative level of the expression of *DFR*, *ANS*, and *3GT* in the M2 mutant versus the control entailed a decline in the total anthocyanin content in the mutant when compared to the control. These results show the direct relationship between the expression levels of these genes and the total anthocyanin content. Previous studies have shown that the type and quality of the synthesized anthocyanin are related to the activity of the *F3'H* and *F3'5'H* genes in the upstream of the anthocyanin biosynthesis pathway (Castellarin and Gaspero 2007). But, the quantity of anthocyanin synthesized is directly related to the *DFR*, *ANS*, and *3GT* expression in the downstream of the anthocyanin biosynthesis pathway in chrysanthemums (Chen *et al.* 2012; He *et al.* 2013). The positive and significant coefficient of correlation between total anthocyanin content and the relative level of *DFR*, *ANS*, and *3GT* expression supports this direct relationship (Table 2).

Table 2. Correlation coefficients between total anthocyanin content and relative expression genes

	Anthocyanin content	*CHS*	*CHI*	*F3H*	*F3'H*	*DFR*	*ANS*
CHS	0.3 ^{ns}	1					
CHI	0.37 ^{ns}	0.39 ^{ns}	1				
F3H	0.52 ^{ns}	0.44 ^{ns}	0.94 **	1			
F3'H	0.56 ^{ns}	0.6 ^{ns}	0.74 **	0.81 **	1		
DFR	0.58 *	0.81**	0.56 ^{ns}	0.64 *	0.4 ^{ns}	1	
ANS	0.74 **	0.64*	0.39 ^{ns}	0.58 *	0.53 ^{ns}	0.76 **	1
3GT	0.81 **	0.46 ^{ns}	0.4 ^{ns}	0.62 *	0.59 *	0.58 *	0.89 **

ns, * and **: represent non-significant and significant at 0.05 and 0.01 levels of probability, respectively.

Based on the results of correlation coefficients, the total anthocyanin content had the highest coefficient of correlation with the relative expression levels of *3GT* ($r = 0.81^{**}$), *ANS* ($r = 0.74^{**}$), and *DFR* ($r = 0.58^{*}$). The result of this positive and significant correlation coefficient was evident in the color of the mutants. The coefficient of correlation between the total anthocyanin content and relative expression level of the other genes of the anthocyanin biosynthesis pathway was not significant. The insignificant relationship between the *F3'H* expression level and the total anthocyanin content ($r = 0.56^{ns}$) shows the role of this gene in determining the type and quality of the synthesized anthocyanin, but it had no significant impact on the total anthocyanin content. Lee et al. (2008) also reported the lack of a relationship between the total anthocyanin content and the *F3'H* expression level in the chrysanthemum.

To select the genes that play a more important role in variations of total anthocyanin content, the stepwise regression analysis was employed in which total anthocyanin content was the dependent variable and the relative expression level of the genes was the independent variables (Table 3).

The results showed that only the relative expression level of *3GT* remained in the model. This gene accounted for 65% of the variations in total anthocyanin content. The significance of the F-statistic at the $p< 0.01$ level revealed that the effect of the relative expression level of *3GT* was significant on total anthocyanin content. Accordingly, the regression equation was defined as $y = 0.027 + 0.81x$ in which *y* represents the total anthocyanin content and *x* represents the relative expression level of *3GT*. The coefficient of regression was estimated at 0.81 and the y-intercept at 0.027. This regression model could capture the variance in the total anthocyanin content with a 99% confidence.

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Table 3. The stepwise regression of total anthocyanin content as dependent variable and relative expression of genes as independent variables

Variable entered	Regression coefficient	Standard error	F
3GT	0.81	0.001	18.4 **
	y-intercept = 0.027	R^2 = 0.65	

Conclusions

The lack of a significant variation in the relative expression levels of *CHS*, *CHI*, and *F3H* in all mutants versus the control and the insignificant coefficient of correlation between total anthocyanin content and relative expression levels of these genes shows that the expression of these genes had no profound impact on the color change of mutants compared to the control. The increased expression of *F3'H* in the upstream of the anthocyanin biosynthesis pathway in the M1 and M3 mutants was accompanied by an increased expression of *DFR*, *ANS*, and *3GT* in the downstream of this pathway. As well, the reduction of the *F3'H* expression resulted in a decrease in the expression of the downstream genes in the M2 mutant. This implies the direct impact of the *F3'H* expression on the expression of other genes in the downstream and consequently, the color change in the mutants versus the control. Since the relative expression levels of *F3'H* were significantly different in all mutants compared to the control and these differences were directly related to the color change of the mutants versus the control, so the *F3'H* gene was introduced by the study as the key gene and it can be used as an appropriate target gene in genetic manipulations. Similarly, He et al (2013) studied the expression of the genes involved in the anthocyanin biosynthesis pathway of the red, pink, and purple genotypes of chrysanthemums and reported that the *F3'H* gene was expressed to a greater extent in the red and purple genotypes than in the pink genotypes, so they proposed this gene as a proper target gene for genetic manipulations. Since the structural genes involved in the anthocyanin biosynthesis pathway are active in white chrysanthemums and their expression is not suppressed on the one hand and the lack of pigmentation of these white chrysanthemums is related to the low activity of these genes on the other hand (Chen *et al.* 2012), it is possible to transfer *F3'H* from our studied samples to the white chrysanthemums to test the likelihood of producing red petals. Although the relative expression level of *F3'H* was higher in the M1 mutant than in the M3 mutant, the relative expression levels of *DFR*, *ANS*, and *3GT* were higher in the M3 mutant than in the M1 mutant, perhaps due to the more activity of *F3'5'H* and the production of the DHM substrate and consequently, the higher activity of the downstream genes of the anthocyanin biosynthesis pathway in the M3 mutant. Accordingly, the M3 mutant is a more suitable sample to study the possibility of producing blue chrysanthemum by suppressing the expression of *F3'H* using the RNAi method. This study showed the effect of the relative expression levels of the structural genes involved in the anthocyanin biosynthesis pathway on the color change of mutants induced by gamma irradiation of purple chrysanthemums 'Rambla' at 40 Gy.

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Effect of gamma irradiation on the expression of structural genes involved in anthocyanin biosynthesis pathway in chrysanthemum

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Abstract

Chrysanthemums (*Chrysanthemum morifolium*) are an important ornamental plants in Iran and the world with a high commercial value. Among different doses of gamma irradiation, the irradiation at 40 Gy by a Cobalt-60 source to four-week-old seedlings of a purple commercial cultivar of chrysanthemum, i.e. 'Rambla' produced three mutants with dark purple, pale purple, and Indian red colors. To explore the effect of variations in the expression levels of the structural genes involved in the anthocyanin biosynthesis pathway on the color of the mutants, the RNA of the mutants and control plants was extracted from the petals of the M1V3 generation and studied by the RT-qPCR method. The significant changes in the relative expression levels of *F3'H*, *DFR*, *ANS*, and *3GT* in the mutants versus the control showed the role of these genes in the petal color change. The total anthocyanin content had significant correlation with the relative expression levels of *DFR*, *ANS*, and *3GT*. Stepwise regression for the total anthocyanin content as the dependent variable and the relative expression level of the structural genes involved in the anthocyanin biosynthesis pathway as independent variables showed that *3GT* had the strongest impact on dependent variable. *F3'H* relative expression caused significant changes in the mutants versus the control and it was introduced as an appropriate key gene for genetic manipulations to create color diversity in chrysanthemums.

Keywords: anthocyanin, chrysanthemum, gamma irradiation, mutant, structural genes.

Abbreviations: *CHS*: Chalcone synthase, *CHI*: Chalcone isomerase, *F3H*: Flavanone 3-hydroxylase, *F3'H*: Flavonoid 3'-hydroxylase, *F3'5'H*: Flavonoid 3'5'-hydroxylase *DFR*: Dihydroflavonol 4-reductase, *ANS*: Anthocyanidin synthase, *3GT*: 3-*O*-glucosyltransferase, DHK: Dihydrokaempferol, DHQ: Dihydroquercetin, DHM: dihydromyricetin, Gy: Gray.

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