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Red Pigment Content and Expression of Genes Related to Anthocyanin Biosynthesis in Radishes (*Raphanus sativus* L.) with Different Colored Flesh

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Abstract

Radish with red skin and red flesh (*Raphanus sativus*) is a unique vegetable containing large amounts of a red pigment, which is widely used in foods, wine, and cosmetics. To investigate the gene or genes that play a key role in anthocyanin biosynthesis in radish with red skin and red flesh, the red pigment content and expression of genes involved in anthocyanin biosynthesis of 16 varieties with different colored flesh were studied. The expression level of the genes *RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, and *RsANS* in radish with red skin and red flesh are all significantly higher than that of radish with white skin and white flesh, radish with red skin and white flesh, radish with green skin and pinky flesh, and radish with red skin and pinky flesh. Correlation analysis indicated that the gene expression level of *RsDFR*, *RsF3H*, *RsCHS*, *RsANS*, *RsF3'H*, *RsCHI*, and *RsPAL* showed remarkable positive and significant correlation to red pigment content of radish. Stepwise regression analysis showed that the gene expression level of *RsDFR* had the highest and significant direct effect (0.8932) on red pigment content of radish. The results indicated that (1) the red pigment content of radish is closely related to the increased expression of a number of structural genes in anthocyanin biosynthesis, (2) the *RsDFR* gene plays a key role in anthocyanin biosynthesis in red radish with red flesh, and (3) *RsDFR* might be one of the best targets in genetic engineering for anthocyanin production from radish and other plants.

Keywords: radish with red skin and red flesh, red pigment, gene expression, correlation analysis, stepwise regression analysis

1. Introduction

Chinese radishes possess the richest genetic resources in the world, which can be further differentiated by fleshy root size, shape, and color, as well as leaves (Wang et al., 2015). Traditionally, Chinese radishes have been classified into five types: radish with white skin and white flesh (Figure 1A), radish with red skin and white flesh (Figure 1B), radish with green skin and pinky flesh (Figure 1C), radish with red skin and pinky flesh (Figure 1D), and radish with red skin and red flesh (Figure 1E) (Chen et al., 2015). Of these types, the radish with red skin and red flesh is a unique ecotype which contains large amounts of a red pigment which is widely used in foods, wine, and cosmetics. A previous study on red radish with red skin and red flesh detected that the red pigment content of radish with red skin and red flesh was 6.4-28.8 ‰ with an average of 16.13 ‰, indicating that the radish with red skin and red flesh is a valuable source of material for the red pigment industry (Chen et al., 2015). The red pigment content and production of radish with red skin and red flesh are eight and four times higher than that of radish with green skin and pinky flesh, respectively (Tong et al., 2013). In recent years, the physiological and biochemical characteristics (Fang et al., 2012), inheritance of traits (Lv et al., 2015) and the extraction and separation of red pigment (Tong et al., 2013) from radish with red skin and red flesh have been well studied. However, despite these studies, little is known about the molecular mechanism of anthocyanin accumulation in radish with red skin and red flesh.

Red pigment is prevalent in plant leaves, petals, and fruits and is a type of water-soluble anthocyanin belonging to the flavonoid. The pigment is associated with the colors of red, blue, purple, red-purple or other colors. As the biosynthesis pathway of anthocyanin in plants has been studied in more detail (Naing et al., 2015), the extraction,

biosynthesis, metabolic pathways, and regulatory mechanisms of anthocyanins have received increased attention. The accumulation of anthocyanin in plants is closely related to the expression level of genes associated with anthocyanin biosynthesis (Zhang et al., 2015). There are two types of genes that control anthocyanin biosynthesis in plants: one is the structural gene that encodes the anthocyanin biosynthetic pathway enzymes, and the other is a regulatory gene that regulates spatiotemporal expression of the structural gene (Rouholamin et al., 2015; Zhao et al., 2015). The chalcone synthase gene (*CHS*) plays an essential role in anthocyanin biosynthesis and regulates the relative amount of specific flavonoids (Zhang et al., 2015). The change of expression in the *CHS* gene can significantly affect anthocyanin accumulation (Yin et al., 2015). The interference decreased *CHS* expression and anthocyanin content by 88.8% and 87% in *Prunus persica*, respectively (Zhang et al., 2015). Overexpression of *FhCHS1* in *Arabidopsis thaliana* changed the pigmentation phenotype of the seed coats, cotyledons, and hypocotyls (Sun et al., 2015). Chalcone isomerase (*CHI*) catalyzes the stereospecific isomerization of chalcones into their corresponding (2S)-flavanones (Guo et al., 2015). *IbCHI* of sweet potato is responsible for the activation of anthocyanin biosynthesis in the early stage of root development (Guo et al., 2015). Flavanone 3-hydroxylase (*F3H*) catalyzes flavanone into flavonol and one to two *F3H* genes were found to be cloned in most plants, and *F3H* gene has two introns with 350-380 amino acids in protein (Laura, 2013; Kumar et al., 2015). Flavonoid 3′-hydroxylase (*F3′H*) and flavonoid 3′5′-hydroxylase (*F3′5′H*), both of which belong to the cytochrome P450 superfamily, catalyze hydroxylation at the 3′ and 3′, 5′ positions of the B-ring of the flavonoids (Forkmann et al., 2014; Nakamura et al., 2015). Dihydroflavonol 4-reductase (*DFR*), only expressed in the colored organ responsible for anthocyanidin production, catalyzes flavanonols into leucoanthocyanidin (Xu et al., 2014). The total anthocyanin content has a positive correlation between the level of *DFR* gene expression and the accumulation of anthocyaninsin in *Punica granatum* (Rouholamin et al., 2015). Anthocyanidin synthase (ANS) catalyzes the conversion of colorless leucoanthocyanins into colored anthocyanins (Shi et al., 2015a). The transcript level of *MsANS* of *Magnolia sprengeri* was 26-fold higher in red petals than in white petals (Shi et al., 2015b). Meanwhile, the anthocyanin accumulation is associated with the expression of several structural genes in the anthocyanin biosynthetic pathway (Zhao et al., 2015b). In *Morus alba*, *MaCHS, MaDFR*, and *MaANS* genes control the anthocyanin biosynthesis in mulberry and upregulation of them greatly increases the anthocyanin content (Li et al., 2014). *CHS* and *DFR* play important roles in the process of the color changes of rose petals from yellow to red (Luo et al., 2013). R2R3-MYB, bHLH, and WD40 proteins are the principal regulatory factors involved in anthocyanin biosynthesis (Shoeva et al., 2015). The combination and interaction amongst the regulatory factors determine the expression of structural genes. The different co-expression of *MYB10* and *bHLH33* genes and the different expressions of WD40 are involved in the differential regulation mechanisms of anthocyanin biosynthesis and the coloration pattern between occidental and oriental pears (Yang et al., 2015). *LcMYB1* controls anthocyanin biosynthesis in litchi, and *LcUFGT* might be the structural gene that is targeted and regulated by *LcMYB1* (Lai et al., 2014). Concerning the anthocyanin biosynthesis in radish, Park et al. (2011) reported that *RsDFR* and *RsANS* were found to accumulate in the flesh or skin, and radish skin contained higher *CHS*, *CHI*, and *F3H* transcript levels than radish flesh. Lim et al. (2015) isolated the *RsMYB1* gene from red radish (red skin) plants and suggested that *RsMYB1* is an actively positive regulator for anthocyanin biosynthesis in radish plants. To summarize, although the mechanism of anthocyanin accumulation in many plants have been reported, little is known about the genes associated with the anthocyanin accumulation in radish with red skin and red flesh because of its specific distribution and the red pigment content is remarkably reduced when the radish be cultivated in other locations.

With the trend of synthetic colorants progressively being replaced by natural colorants, the need for natural pigments has dramatically increased. As a unique agricultural resource, there is a great prospect for the application of radish with red skin and red flesh. Hence, it is necessary to study the molecular mechanisms of anthocyanin accumulation in radish with red skin and red flesh. In this study, the red pigment content, expression of related genes in anthocyanin biosynthesis of 16 radish (*Raphanus sativus* L.) varieties with different colored flesh, and the relationships between red pigment content and gene expression level were studied. The goal of this study was to estimate the key gene or genes associated with anthocyanin biosynthesis for future study of the mechanisms of anthocyanin accumulation in radish with red skin red flesh.

2. Materials and Methods

2.1 Plant Materials

In total, 16 radish varieties were sampled, including four radishes white skin and white flesh, three radishes with red skin and white flesh, one radish with green skin and pinky flesh, five radishes with red skin and pinky flesh, and three radishes with red skin and red flesh (Table 1 and Figure 1). The seeds were abtained from Crop Genetics and Breeding Research Center, Yangtze Normal University, China.

2.2 Radish Red Pigment Content Measurements

A field trial with randomized complete block design was completed at the farmland of the Yangtze Normal University, China. The experiments were carried out with three replications. Each accession was planted in a single row of 30 plants with 40 cm between rows and 30 cm between plants. Before bolting, 15 representative plants were sampled to measure red pigment content of the radishes. For each sample, the skin and flesh were mixed together, and the root flesh was divided into two parts; one part was used to determine the water content, and juice was extracted from the other one. After centrifuging the juice at 4000×g for 10 min, the absorbance of the supernatant at 520 nm was determined by spectrophotometry. After the juices were separated by high-performance liquid chromatography (HPLC) (LC-2010CHT, Shimadzu Ltd., Kyoto, Japan) the red pigment content was determined by a UV-Vis scanning spectrophotometer (U-3010, Hitachi Seiki Co. Ltd., Abiko, Japan) using the standard curve method (Chen et al., 2015). The red pigment content (‰) = Total pigment weight (g)/ Total root flesh weight (g) ×1000.

Table 1. The materials used in this study and their characteristics

Code	Sources	Leaf shape	Leaf color	Leafstalk color	Root skin color	Flesh color	Red pigment content ‰
1	Suzhou, Zhejiang	strip-shaped	green	green	white	white	0.21
2	Mianyang, Sichuan	flower-shaped	green	green	white	white	0.03
3	Mianyang, Sichuan	flower-shaped	green	green	white	white	0.01
4	Mingquan, Henan	flower-shaped	green	green	white	white	0.01
5	Mianyang, Sichuan	strip-shaped	green	light red	light red	white	2.41
6	Chengdu, Sichuan	flower-shaped	green	light red	light red	white	1.25
7	Mingquan, Henan	flower-shaped	dark green	light red	light red	white	2.57
8	Yutian, Hebei	flower-shaped	green	green	green	pinky	4.53
9	Fuling, Chongqing	flower-shaped	light green	red	red	pinky	11.23
10	Fuling, Chongqing	strip-shaped	dark green	red and green	red	pinky	10.58
11	Fuling, Chongqing	flower-shaped	green	red and green	red	pinky	8.68
12	Fuling, Chongqing	flower-shaped	green	red and green	red	pinky	7.26
13	Fuling, Chongqing	flower-shaped	green	light red	red	pinky	8.34
14	Fuling, Chongqing	flower-shaped	green	dark red	dark red	red	25.43
15	Fuling, Chongqing	strip-shaped	green	dark red	dark red	red	23.48
16	Fuling, Chongqing	flower-shaped	dark green	dark red	dark red	red	22.58

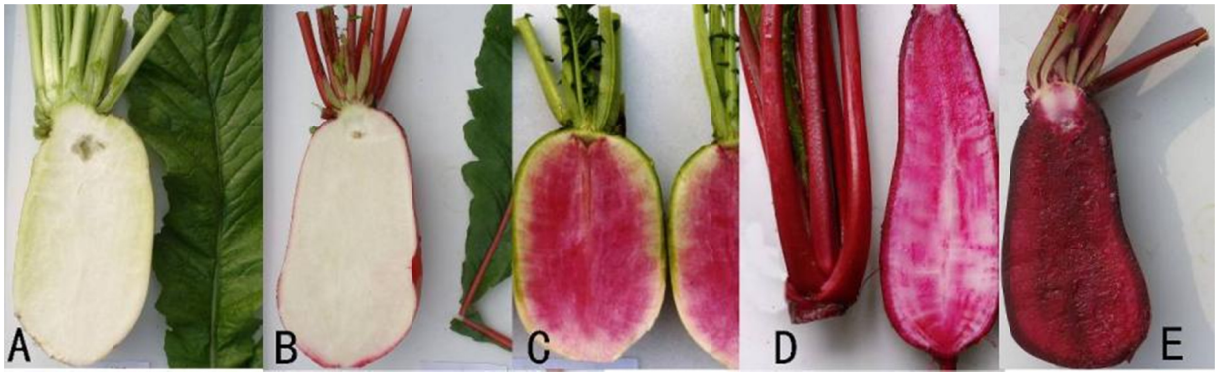


Figure 1. Different types of radishes

Note. A: radish with white skin and white flesh, B: radish with red skin and white flesh, C: radish with green skin and pinky flesh, D: radish with red skin and pinky flesh, and E: radish with red skin and red flesh.

2.3 RNA Extraction and Reverse Transcription

Total genomic RNA was extracted from the flesh of the root before bolting using a Column Plant Total RNA Extraction Kit SK8661 (Sangon Biotech Shanghai Co. Ltd., Shanghai, China). Agarose electrophoresis and spectrophotometry were used to determine the quality and content of total RNA, respectively. The cDNA were acquired by using an AMV First Strand cDNA Synthesis Kit SK2445 (Sangon Biotech Shanghai Co. Ltd., Shanghai, China). The first strand synthesis of cDNA was performed in a 0.2 mL PCR tube containing 5 μL of total RNA, 1 μL of random primer $p(dN)_6$ (0.2 μg/μL), and 5 μL of RNase-free ddH_2O. The mixed liquor was measured with thermostatic bath at 70 °C for 5 min, followed by freezing for 10 sec. After centrifugation, 4 μL of 5 × reaction buffer, 2.0 μL of dNTP mix (10 mmol/L), 1.0 μL of RNase inhibitor (20 U/μL), and 2.0 μL AMV reverse transcriptase (10 U/μL) were added to the PCR tube. After a thermostatic bath at 37 °C for 5 min followed by 42 °C for 60 min, the reaction was stopped in the thermostatic bath at 70 °C for 10 min. The mixtures were stored at -20 °C for post-study.

2.4 Fluorescence Quantitative PCR Analysis

The primers for radish *RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, *RsANS*, and *RsMYB* were designed from the conserved sequences of known orthologous sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The primers were synthesized by Sangon Biotech (Shanghai, China), and are listed in Table 2. The fluorescence quantitative PCR was performed in a reaction mixture (20 μL) containing 10 μL of 2 × ABI SybrGreen PCR Master Mix (Sangon Biotech Shanghai Co. Ltd., Shanghai, China), 1 μL of each primer (10 μmol/mL), 2 μL (50 ng) of cDNA, and 7 μL of ddH_2O. The ABI StepOne Plus™ System (Applied Biosystems, USA) was programmed to run for 2 min at 95 °C at the initial step, followed by 40 cycles of melting for 10 sec at 95 °C, then annealing and extending for 40 sec at 60 °C. The fluorescence quantitative PCR experiments were performed with three replications for each sample.

Table 2. The primers designed from the homologous sequences

Name	Forward primer	T_m	Reverse Primer	T_m	Product length
actin	TATGAGCAAAGAGATCACAGCACT	58.8	TGAGGGAAGCAAGAATGGAA	58	113
RsPAL	CATGTTCGCTCAGTTCTCCG	58.9	TCAGCTCCTTTGAATCCGTAA	57.7	111
RsCHS	GCAGGGATTTTGGCGATAG	58.2	CTGTTGGTGATGCGGAAGTAG	58.2	89
RsCHI	GTGCTTCCATCCTCTTCGCT	59.2	AACTGCCTCTGCCAACAACT	57.2	122
RsDFR	TCATCGGTTCATGGCTCGT	59.1	CCGTTTATGGCGTCATCGTA	59.5	184
RsF3H	GGTGGGTGAAAGTGACGGA	58.4	GCTGAGGGCATTTTGGGTA	58.3	168
RsF3'H	CAAACACGAAACCAGTGAACC	57.9	AGCGACGCCTTGTAAATCTAA	57.5	218
RsANS	TACATTGAAGCGACGAGTGAGT	57.6	AGGGCATTTCGGGTAATAGTT	57.7	171
RsMYB	GATAAGTATGGAGAAGGCAAATGG	59.5	TCAGCCATCTTAGTCTACAACTCTTC	58.8	88

2.5 Statistical Analyses

Variance analysis was carried out to evaluate differences between radish varieties for red pigment content and gene expression with SPSS software (Verma, 2013) followed by multiple comparisons using Duncan's new multiple range method. When significant treatment differences occurred, the correlation coefficients among red pigment content and gene expression and stepwise regression analysis were also calculated in this program.

3. Results

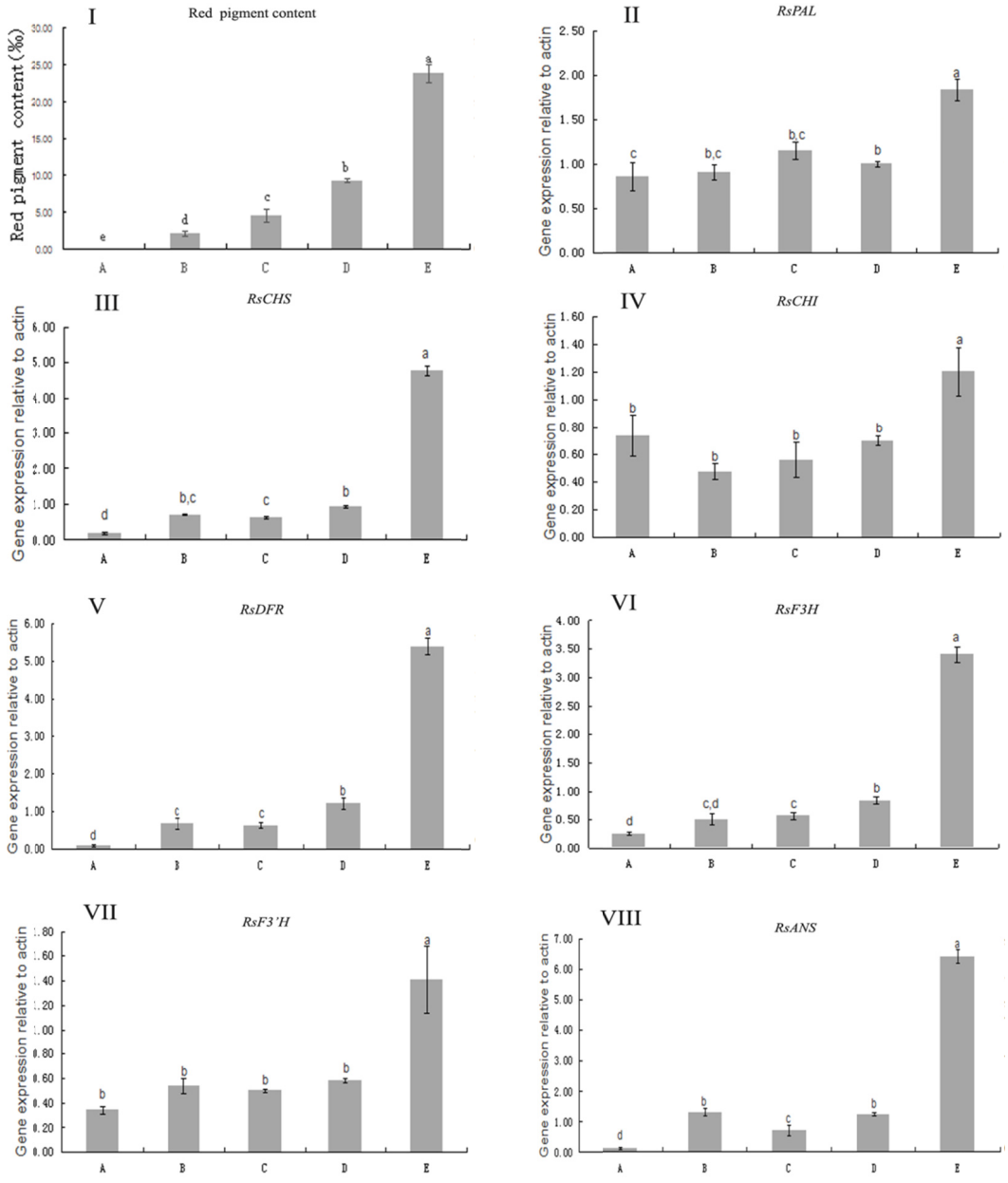
3.1 Differences in Genes Expression with Different Colored Flesh

The results of the F-test of gene expression in 16 radishes are listed in Table 3. It is shown that the variance of gene expression level is significant at the 1% level in the *RsPAL*, *RsCHS*, *RsDFR*, *RsF3H*, *RsF3'H*, *RsANS* and *RsMYB* genes and significant at the 5% level in the *RsCHI* gene. The average gene expression level of *RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, *RsANS* and *RsMYB* genes are 1.12, 1.40, 0.75, 1.57, 1.10, 0.66, 1.91, and 2.14, respectively. It was indicated that the expression level of *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, and *RsANS* genes in radish with red skin and red flesh were all highest, while the expression level of *RsPAL*, *RsCHS*, *RsDFR*, *RsF3H*, *RsF3'H*, and *RsANS* genes in radish with white skin and white flesh are all lowest.

Table 3. Variance analysis for genes expression

Genes	Mean	Range	DF			MS			F-Value	
			Block	Treatment	Error	Block	Treatment	Error	Block	Treatment
RsPAL	1.12	0.51-2.91	2	15	30	0.30	1.03	0.07	4.03*	13.95**
RsCHS	1.40	0.10-6.43	2	15	30	0.03	11.43	0.18	0.15	62.32**
RsCHI	0.75	0.28-1.33	2	15	30	0.03	0.31	0.13	0.22	2.35*
RsDFR	1.57	0.08-6.64	2	15	30	0.54	12.84	0.58	0.94	22.19**
RsF3H	1.10	0.16-4.58	2	15	30	0.11	4.96	0.08	1.49	65.75**
RsF3'H	0.66	0.23-1.97	2	15	30	0.24	0.67	0.08	3.01	8.45**
RsANS	1.91	0.09-9.17	2	15	30	0.07	20.66	0.22	0.33	95.98**
RsMYB	2.14	0.45-14.56	2	15	30	1.34	35.19	0.73	1.84	48.44**

Note. * and ** represent the significant levels at 0.05 and 0.01, respectively.



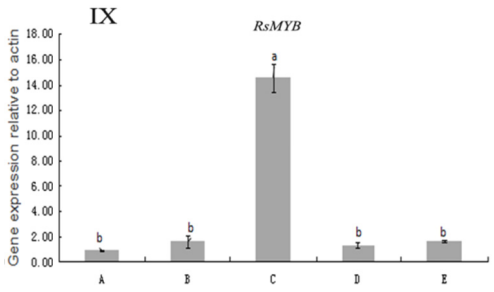


Figure 2. Red pigment content and expression of related anthocyanin biosynthesis genes in five types of radishes

Note. (I): Mean of red pigment content for the five types of radishes. (II) *RsPAL*, (III) *RsCHS*, (IV) *RsCHI*, (V) *RsDFR*, (VI) *RsF3H*, (VII) *RsF3'H*, (VIII) *RsANS* and (IX) *RsMYB* gene expression in flesh root for the five radish types. A: radish with white skin and white flesh, B: radish with red skin and white flesh, C: radish with green skin and pinky flesh, D: radish with red skin and pinky flesh, and E: radish with red skin and red flesh. Error bars represent the standard error of the mean (n = 3). Variance analysis was carried out to evaluate differences between radishes for red pigment content and gene expression followed by multiple comparisons using Duncan's new multiple range method. Different letters indicate significance at the P = 0.05 level.

As far as the genes expression of the five types of radishes are compared (Figure 2, II to IX), the average gene expression level of *RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, and *RsANS* in radish with red skin and red flesh are significantly higher than the other four types of radishes. The average gene expression level of *RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, and *RsANS* in radish with red skin and red flesh are 1.15-, 24.78-, 0.63-, 57.99-, 12.31-, 3.10-, and 49.77-fold higher than in radish with white skin and white flesh. The average gene expression level of the *RsMYB* gene in radish with green skin and pinky flesh is significantly higher than in the other four types of radishes.

3.2 Differences in Red Pigment Content between Radishes with Different Colored Flesh

The ANOVA F-test in the SPSS software was used to identify significant differences in red pigment content among radish varieties with different colored flesh. As shown in Table 1, the red pigment content of 16 radish varieties with different colored flesh ranged from 0.01 to 25.43‰ with an average of 8.04‰. Significant differences were found among the 16 radishes (Table 4, F = 114.29, P = 0.0001). The red pigment content differed very markedly in different types of radish varieties (Figure 2, I). The radish with red skin and red fresh had the highest red pigment content with an average of 23.83 ‰, followed by radish with red skin and pinky flesh (9.22‰), radish with green skin and pinky flesh (4.53‰), radish with red skin and white flesh (2.08‰), and radish with white skin and white flesh (0.07‰). As shown in Figure 2, the red pigment content of radish with red skin and red fresh is significantly higher than that of the other four types of radishes. It indicates that real differences existed in these radishes with red pigment content.

Table 4. Variance analysis for red pigment content of radishes

Source of variation	Squares	Df	MS	F value	P value
Block	5.81	2	2.91	1.41	0.2598
Treatment	3531.66	15	235.44	114.29	0.0001
Error	61.80	30	2.06		
Totall variation	3599.27	47			

3.3 Correlation Analysis among Red Pigment Content and Gene Expression

Table 5. Correlation analysis among red pigment content and gene expression in the 16 radishes

	RsCHS	*RsCHI*	*RsDFR*	*RsF3H*	*RsF3'H*	*RsANS*	*RsMYB*	Red pigment
RsPAL	0.67**	0.52*	0.67**	0.58*	0.44	0.61**	0.00	0.57*
RsCHS		0.68**	0.92**	0.98**	0.87**	0.98**	-0.06	0.83**
RsCHI			0.76**	0.73**	0.66**	0.67**	-0.18	0.64**
RsDFR				0.95**	0.84**	0.92**	-0.08	0.89**
RsF3H					0.93**	0.98**	-0.07	0.88**
RsF3'H						0.92**	-0.10	0.80**
RsANS							-0.09	0.83**
RsMYB								-0.04

Note. * and ** represent the significant levels at 0.05 and 0.01, respectively.

In order to better determine the relationship between the related gene expression and the red pigment content of radishes, we carried out a correlation analysis with the red pigment content considered as the dependent variable and the gene expression level of *RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, *RsANS* and *RsMYB* genes considered as the independent variable. Correlation analysis (Table 5, coefficient of determination $R^2 = 0.9335$) indicated that the correlation coefficients of the gene expression level to the red pigment content, in order of large to small, were *RsDFR*, *RsF3H*, *RsCHS*, *RsANS*, *RsF3'H*, *RsCHI*, *RsPAL*, and *RsMYB*. Among the correlation coefficients, the gene expression level of *RsDFR*, *RsF3H*, *RsCHS*, *RsANS*, *RsF3'H*, *RsCHI*, and *RsPAL* showed positive remarkable significant correlation to red pigment content, and the gene expression level of *RsPAL* showed positive significant correlation to red pigment content. The gene expression level of *RsMYB* showed negative and insignificant correlation to red pigment content. Moreover, 20 correlation coefficients among the gene expression level were determined to be significant.

3.3 Path Correlation Analysis among Red Pigment Content and Gene Expression

Correlation analysis revealed that there are seven genes that show a significant correlation to red pigment content, indicating that the red pigment content trait is controlled by multigenes and that the relationship among the gene expression levels are close. Therefore, stepwise regression analysis was carried to further determine the importance of these gene expressions on red pigment content. Stepwise regression analysis indicated that the contribution frequency of *RsDFR* gene expression level (X_4) to red pigment content is 87.23%. The regression equation is as follows: $Y = 2.10 + 3.77\ X_4$ ($t = 7.4331$, $p = 0.0001$). The gene expression level of *RsDFR* had the highest and significant direct effect (0.8932) on red pigment content of radish.

4. Discussion

Radish with red skin and red flesh, an intense source of red pigment, has been the subject of much scientific interest owing to its large amount of a red pigment that is widely used in foods, wine, and cosmetics. Radish with red skin and red flesh is indigenous to the city of Chongqing, and the red pigment content is remarkably reduced when cultivated in other locations. The data on the molecular mechanism of anthocyanin accumulation in radish with red skin and red flesh can be applied to better protecting and using the resource, and also for genetic engineering breeding which may lead to the creation of new radish materials. Although the mechanism of anthocyanin accumulation in some plants has been studied extensively, little is known about the molecular mechanism of anthocyanin accumulation in radish with red skin and red flesh. Investigations have revealed that the accumulation of anthocyanin in plants is controlled by two types of genes and is closely related to the expression level of genes associated with anthocyanin biosynthesis (Zhao et al., 2015a). Our research showed that the average gene expression level of *RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, and *RsANS* in red radish with red flesh are significantly higher than in four other types of radishes, and the expression level of these genes all showed positive significant correlation to red pigment content. The results indicated that the red pigment content is closely related to the expression level of genes associated with anthocyanin biosynthesis. The results were consistent with the previously reported data by Louarn et al. (2007) that anthocyanin accumulation coincides with a coordinated increase in the expression of a number of structural genes (*RsPAL*, *RsCHS*, *RsCHI*, *RsDFR*, *RsF3H*, *RsF3'H*, and *RsANS*) in the anthocyanin biosynthetic pathway. However, the gene expression

level of the regulatory gene (*RsMYB*) in radish with green skin and pinky flesh is significantly higher than in the other four types of radishes, and there is no insignificance among the other four types, while the gene expression level of *RsMYB* showed negative and insignificant correlation to red pigment content. By contrast, Lim et al. (2015) suggested that *RsMYB* is an actively positive regulator for anthocyanins biosynthesis in radish plants (radish with red skin and pinky flesh), Zhao et al. (2015b) reported that both *RhMYBs4-1* and *RhMYBs6-1* were highly expressed in red petals and might be important regulators of anthocyanin biosynthesis and coloration in rose petals. It may be due to the fact that the regulation mechanism of the *RsMYB* genes is different.

Previous studies suggest that the key genes controlling anthocyanin accumulation in plants are different. Zhang et al. (2015), Yin et al. (2015), and Sun et al. (2015) suggested that the *CHS* gene plays an essential role in anthocyanin biosynthesis. Guo et al. (2015) suggest that *IbCHI* is a key enzyme in the in the anthocyanin biosynthesis of sweet potato. Rouholamin et al. (2015) found that total anthocyanin content measurement showed a positive correlation between the level of *DFR* gene expression and accumulation of anthocyanins in different genotypes in *Punica granatum*. Shi et al. (2015b) reported that the transcript level of *MsANS* of *Magnolia sprengeri* was 26-fold higher in red petals than in white petals. Li et al. (2014) suggested that *MaCHS*, *MaDFR*, and *MaANS* genes may control the anthocyanin biosynthesis in mulberry and up-regulation of them may greatly increase the anthocyanin content in *Morus alba*. Zhao et al. (2015a) suggested that the onset of anthocyanin synthesis during berry development coincides with a coordinated increase in the expression of a number of genes (such as *F3'HPAL*, *CHI1*, and *DFR*) in the anthocyanin biosynthetic pathway. Luo et al. (2013) proved that the genes *CHS* and *DFR* play important roles in the process of anthocyanin synthesis. Our data also showed that gene expression level of *RsDFR*, *RsF3H*, *RsCHS*, *RsANS*, *RsF3'H*, *RsCHI*, and *RsPAL* showed positive significant correlation to red pigment content. Meanwhile, most of the correlation coefficients among the gene expression level of the seven genes appeared to be significant. Therefore, stepwise regression analysis was carried out to further determine the importance of the expression of these genes on red pigment content. It is interesting that the contribution frequency of the *RsDFR* gene expression level to red pigment content is 87.23% and the gene expression level of *RsDFR* had the highest and significant direct effect (0.8932) on red pigment content of radish. The average gene expression level of *RsDFR* in radish with red skin and red flesh is 57.99-fold higher than in radish with white skin and white flesh. We hypothesized that the *RsDFR* gene plays a key role in anthocyanin biosynthesis in radish with red skin and red flesh. *RsDFR* might be one of the best targets for anthocyanin production by single gene manipulation that is applicable in radish and other plants.

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