



Article

Combined Metabolomic and Transcriptomic Analysis Reveals Candidate Genes for Anthocyanin Accumulation in *Ginkgo biloba* Seed Exocarp

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Abstract: Anthocyanin is an important pigment that affects plant color change. In this study, the color parameters and anthocyanin content of *Ginkgo biloba* seed exocarp at different periods were measured, and it was determined that the a^* value (redness value) of the seed exocarp was closely related to the color change occurring during the development of the seed exocarp, and the anthocyanin content in the seed exocarp showed an increasing trend. The molecular mechanism of anthocyanin biosynthesis in *Ginkgo biloba* seed exocarp is still unclear. In order to further understand the molecular mechanism of color change in *Ginkgo biloba* seed exocarp, the regulation mechanism and accumulation mode of anthocyanin in the seed exocarp at three different periods were analyzed using transcriptomic and metabolomic. A total of four key anthocyanins were screened from the metabolome, including three kinds of Cyanidin 3-arabinoside, Malvidin 3-glucoside and Cyanidin 3-sambubioside 5-glucoside with increased content. Among them, Cyanidin 3-arabinoside had a strong correlation with the a^* value ($PCC = 0.914$), which have a great influence on the color change of the seed exocarp, and Delphinidin 3-O-3'',6''-O-dimalonylglucoside with decreased content might jointly affect the formation of exocarp color. The transcriptome data show that among the structural genes, *ANS* (*Gb_33402*) had the highest correlation with Cyanidin 3-arabinoside ($PCC = 0.9217$) and in *GbANS*, only *Gb_33402* showed an upregulated expression trend in the three stages of seed exocarp development, which suggesting that it plays an important role in anthocyanin accumulation in the seed exocarp and it may be the key structural gene affecting the formation of seed exocarp color. Among the transcription factors, the differential expression of most transcription factors (*MYB*, *bHLH*, *b-ZIP*, *NAC*, *WDR* and *AP2/ERF*) may jointly affect the formation of seed exocarp color by promoting anthocyanin accumulation. This study elucidates the main anthocyanins that cause the color change of the seed exocarp of *Ginkgo biloba* and reveals the molecular regulation mechanism of anthocyanins at different developmental stages of the seed exocarp. It provides a theoretical basis and insights for understanding the color change of *Ginkgo biloba* seed exocarp.

Keywords: *Ginkgo biloba* L.; anthocyanins; metabolomics; transcriptomics; seed exocarp color



Citation: Tang, J.; Feng, Z.; Xiang, X.; Wang, Y.; Li, M. Combined Metabolomic and Transcriptomic Analysis Reveals Candidate Genes for Anthocyanin Accumulation in *Ginkgo biloba* Seed Exocarp. *Horticulturae* **2024**, *10*, 540. <https://doi.org/10.3390/horticulturae10060540>

Academic Editors: Costas Delis and Jiri Gruz

Received: 6 April 2024

Revised: 3 May 2024

Accepted: 17 May 2024

Published: 22 May 2024



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1. Introduction

Ginkgo biloba L., the oldest relict gymnosperm among existing seed plants, is known as a “living fossil” and “botanical panda” [1]. *Ginkgo biloba* seed exocarp is the fleshy seed coat part of the whole *Ginkgo biloba* seed, which is also the most important quality part of the seed, accounting for about 70% of the whole seed. *Ginkgo biloba* is widely planted in China, and the annual output of *Ginkgo biloba* seed exocarp can reach 180,000 tons. Due to the lack of suitable treatment methods, most of the *Ginkgo biloba* seed exocarp can only be treated as production waste, which is a serious waste of *Ginkgo biloba* resources. Moreover,

it causes pressure on the environment and pollutes soil and water [2]. Therefore, it is of great significance to study the rational development and utilization of *Ginkgo biloba* seed exocarp.

Ginkgo biloba exocarp extract (GBEE) mainly contains *Ginkgo biloba* phenolic acid, *Ginkgo biloba* flavone, *Ginkgo biloba* lactone, *Ginkgo biloba* polysaccharide and other chemical components, among which *Ginkgo biloba* phenolic acid and *Ginkgo biloba* flavone have anti-cancer, antibacterial and anti-allergic effects, and *Ginkgo biloba* lactone and *Ginkgo biloba* polysaccharide have antitumor effects [3,4]. In addition, the outer seed bark of *Ginkgo biloba* can be used to prepare biopesticides in agriculture, make wine, prepare health drinks, make *Ginkgo biloba* paste in food, etc. [5,6]. Therefore, the exocarp of *Ginkgo biloba* is of great research value, and its contribution to medicine, agriculture and food is of great significance.

Anthocyanins are a class of natural water-soluble pigments with important physiological activities, belonging to polyphenolic flavonoid substances, which are widely found in seed plants. Due to their complex and diverse structural modifications, various anthocyanin derivatives are formed to cause changes in plant color [7–9], which can cause changes in the color of flowers, leaves, fruits, seeds and other tissues and organs [10–20]. They can also improve the ability of plants to resist adverse environments. In addition, they are widely used in food, medicine, cosmetics and other industries [21–23]. More than 600 anthocyanins have been identified in nature, among which cyanidin, pelargonidin, peonidin, petunidin, delphinidin and malvidin are the six most common anthocyanins in the plant kingdom [24]. Anthocyanin biosynthesis is a branch of the flavonoid metabolic pathway. The key structural genes of this pathway mainly include chalcone synthetase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonoid 3'-hydroxylase (*F3'H*), flavonoid 3',5'-hydroxylase (*F3'5'H*), dihydroflavonol-4-reductase (*DFR*), anthocyanin synthetase (*ANS*) and Flavonoid glucosyltransferase (*UFGT*) [25]. In recent years, anthocyanin biosynthesis genes have been identified, including *CHS*, *F3'H*, *ANS* and *UFGT*, and the expression levels of these genes have been found to be closely related to anthocyanin accumulation [26–28].

Transcription regulators influence anthocyanin biosynthesis by regulating the expression of structural genes. *MYB* is considered to be a transcription factor that plays a major decisive role in the regulation of anthocyanin biosynthesis [29]. In addition, *bHLH* and *WDR* are also important in the process of anthocyanin synthesis, and they can co-form an MBW transcription factor complex with *MYB* to coordinate the regulation of anthocyanin biosynthesis [30]. During the transformation of *Zanthoxylum* peel from green to red, the *R2R3-MYB* transcription factor *c80935* is highly similar to *MdMYB10* of apple, which can activate downstream *DFR* gene expression, and *c226097* can activate *DFR* and *ANS* promoters, thus promoting anthocyanin synthesis and making the peel red [16]. In red kiwi varieties, *AcbHLH42* and *AcMYB123* can activate the promoters of *AcANS* and *AcF3GT1* and regulate the synthesis of the endopeel tissue-specific anthocyanins of kiwi [31]. The heterologous expression of the *WD40* gene *FtWD40* in Tartary buckwheat in tobacco can significantly increase petal color and anthocyanin content, and both *DFR* and *ANS* related to anthocyanin synthesis are upregulated [32]. In addition, a small number of studies have shown that transcription factors, such as *WRKY*, *NAC* and *HD-zip*, can regulate anthocyanin biosynthesis [33–35].

After female *Ginkgo biloba* trees are pollinated in mid-April each year, the seeds will develop and mature in the following May to October and show the phenomenon of color change from green to yellow to orange-red during the development process. At present, the molecular regulation mechanism of the color change of *Ginkgo biloba* seed exocarp is still unclear. In this study, the color parameters and anthocyanin content of *Ginkgo biloba* seed exocarp were determined, and the key metabolites, structural genes and transcription factors that control the formation of color in *Ginkgo biloba* seed exocarp were studied by metabolomic and transcriptomic analysis. The expression levels of genes were further verified by real-time fluorescence quantitative polymerase chain reaction (qRT-PCR). The results of this study provide a theoretical basis and new insights into the

coloration mechanism of *Ginkgo biloba* seed exocarp and reveal a series of candidate genes for cultivating anthocyanin-rich seed exocarp.

2. Results

2.1. Observation of Growth and Development Phenotype of *Ginkgo biloba* Seed Exocarp and Determination of Color Parameters

In this study, we observed the phenotypes of *Ginkgo biloba* seed exocarp at different growth and development stages. The results show that *Ginkgo biloba* seed exocarp presented a green phenotype in the peak growth period (May–June), while the color changed to yellow in the later growth period (July–August) until the maturation period of the exocarp (September–October) when the outer seed coat turned a distinct orange-red color (Figure 1A).

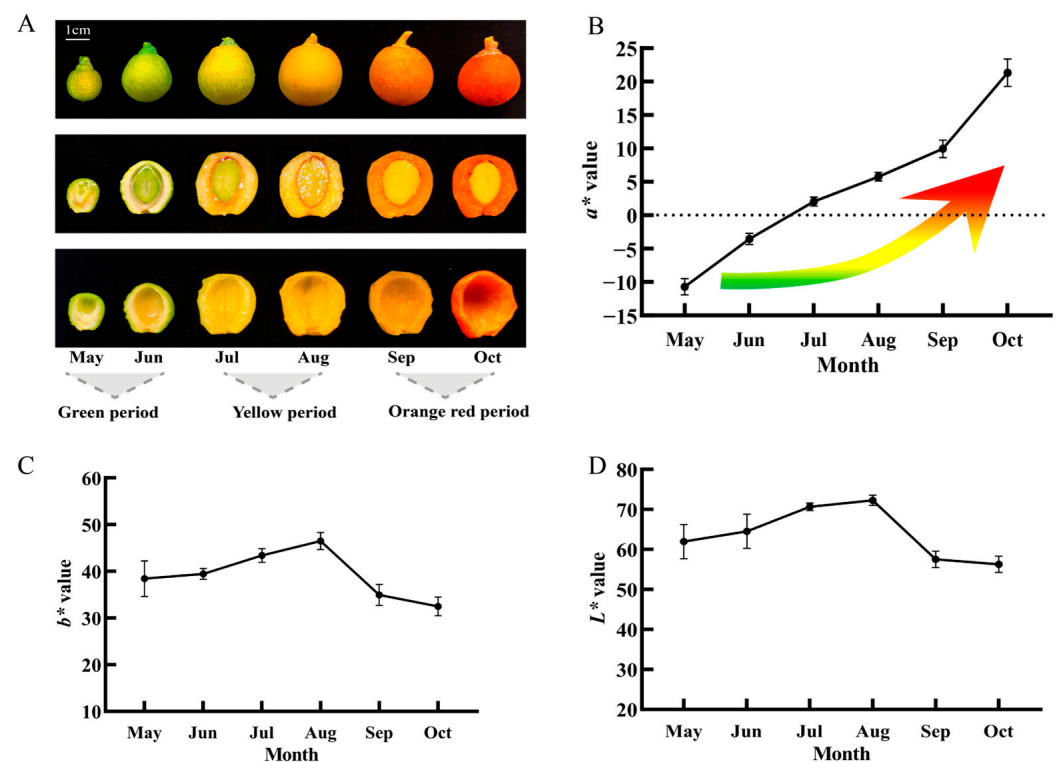


Figure 1. Phenotype observation and color parameter determination of *Ginkgo biloba* seed exocarp. (A) Phenotypic observation of *Ginkgo biloba* seed exocarp at different developmental stages. (B) Redness change trend. (C) Yellowness change trend. (D) Brightness change trend.

In addition, we used a spectrophotometer to measure the color parameters of *Ginkgo biloba* seed exocarp (Table 1). The results show that only the redness (a^*) showed an upward trend with the change in color of the *Ginkgo biloba* seed exocarp (Figure 1B), indicating that the formation of *Ginkgo biloba* seed exocarp color is closely related to the redness. However, yellowness (b^*) and lightness (L^*) showed no significant change trend in different periods (Figure 1C,D), but their change trend was consistent and had a certain correlation, indicating that the degree of cell color in the seed exocarp of *Ginkgo biloba* may be mainly affected by yellowness.

Table 1. Color parameters and anthocyanin content of *Ginkgo biloba* seed exocarp at different developmental stages.

Developmental Stage	Picking Period	CIEL*a*b*			Anthocyanin Content (mg/g)
		Brightness (L*)	Redness (a*)	Yellowness (b*)	
Green period	May	61.96 ± 4.29 bc	−10.70 ± 1.22 f	38.42 ± 3.83 cd	0.1896 ± 0.0304 d
	Jun	64.55 ± 4.27 b	−3.55 ± 0.83 e	39.44 ± 1.16 bc	0.2540 ± 0.0188 bc
Yellow period	Jul	70.69 ± 0.94 a	2.05 ± 0.67 d	43.38 ± 1.46 ab	0.2373 ± 0.0140 c
	Aug	72.29 ± 1.29 a	5.76 ± 0.65 c	46.48 ± 1.83 a	0.2620 ± 0.0129 bc
Orange-red period	Sep	57.53 ± 2.03 cd	9.94 ± 1.32 b	34.94 ± 2.23 de	0.2867 ± 0.0225 b
	Oct	56.29 ± 2.01 d	21.33 ± 2.04 a	32.48 ± 1.99 e	0.3569 ± 0.0275 a

Note: The data in the table are mean ± standard deviation, and different letters indicate significant differences at the $p < 0.05$ level.

2.2. Determination of Anthocyanin Content in *Ginkgo biloba* Seed Exocarp

The anthocyanin (AN) content in the seed exocarp of *Ginkgo biloba* was determined (Table 1). The results show that the content of anthocyanin (AN) was the highest during the orange-red period (September–October), and the average content reached 0.32 mg/g during September–October. The total anthocyanin average content in the yellow period and the green period was 1.29 times and 1.45 times, respectively; the total anthocyanin average content in the yellow period (July–August) was 0.25 mg/g, 1.14 times that in the green period; and the overall total anthocyanin average content in the green period was the lowest at 0.22 mg/g (Figure 2). In addition, the overall change trend of total anthocyanin content was most consistent with that of the seed exocarp. The correlation analysis results show (Table 2) that there was a significant correlation between total anthocyanin and exocarp redness (a^*), and the PCC reached 0.883, which further indicates that anthocyanin plays a leading role in the process of color change in *Ginkgo biloba* seed exocarp. The PCC of yellowness and lightness reached 0.862, which showed a very significant correlation and further confirmed that the brightness degree of *Ginkgo biloba* seed exocarp at different periods was mainly affected by yellowness.

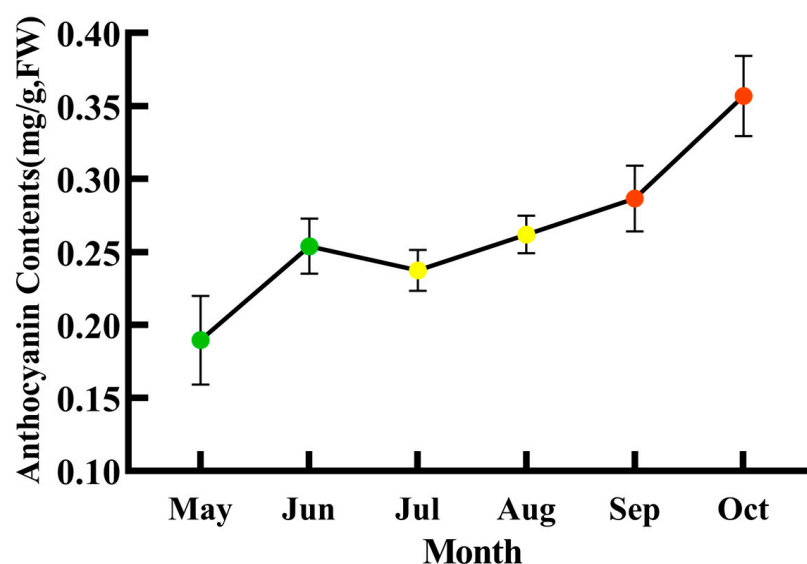
**Figure 2.** Anthocyanin content of *Ginkgo biloba* seed exocarp at different developmental stages.

Table 2. Correlation analysis between color and anthocyanins of *Ginkgo biloba* seed exocarp.

Index	<i>L</i> *	<i>a</i> *	<i>b</i> *	AN
<i>L</i> *	1			
<i>a</i> *	−0.379	1		
<i>b</i> *	0.862 **	−0.402	1	
AN	−0.428	0.883 **	−0.509 *	1

Note: ** Significant correlation at 0.01 level; * Significant correlation at 0.05 level.

2.3. Metabolome Analysis of *Ginkgo biloba* Seed Exocarp in Three Different Color Periods

The anthocyanin data of the metabolome of *Ginkgo biloba* seed exocarp during the green period (GP), yellow period (YP) and orange-red period (ORP) were analyzed. A total of 16 anthocyanins related to color formation were screened (Figure 3).

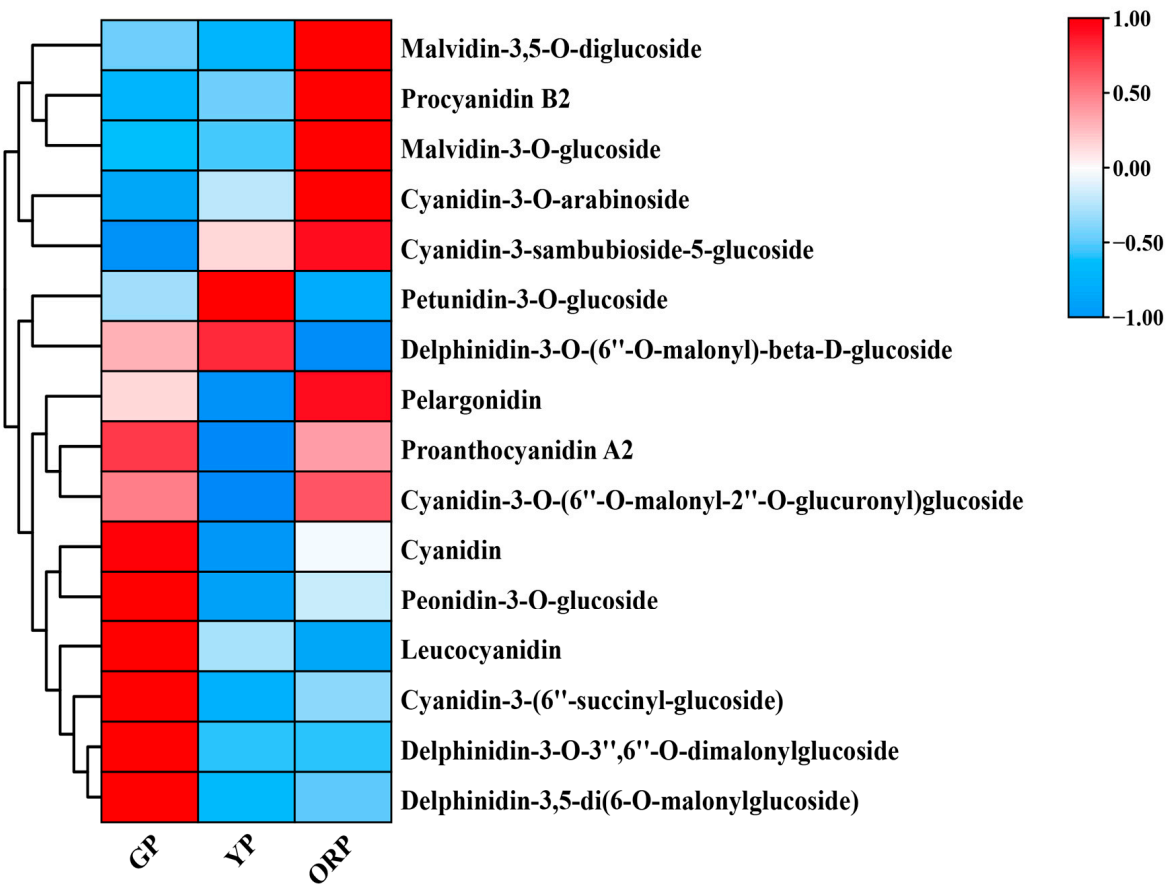


Figure 3. Anthocyanin clustering heat maps of the metabolome of *Ginkgo exocarp* seed exocarp at different periods.

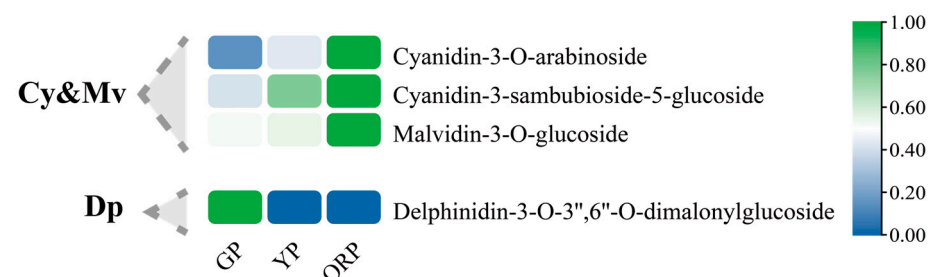
We carried out correlation analysis on the 16 screened anthocyanins and seed exocarp redness (Table 3), and the results show that Cyanidin 3-arabinoside had a very significant positive correlation with exocarp redness, and the correlation reached 0.914. Malvidin 3-glucoside and Cyanidin 3-sambubioside 5-glucoside followed with 0.783 and 0.736, respectively. Therefore, these three anthocyanins were identified as the key positive correlation anthocyanins for seed exocarp discoloration. However, Delphinidin 3-O-3'',6''-O-dimalonylglucoside was significantly negatively correlated with exocarp redness (PCC = −0.743) and identified as a negatively correlated anthocyanin affecting exocarp discoloration.

Table 3. Correlation analysis between the redness of *Ginkgo biloba* seed exocarp and key anthocyanin metabolites.

	<i>a</i> *
Proanthocyanidin A2	−0.026
Leucocyanidin	−0.163
Procyanidin B2	0.572 *
Cyanidin	−0.040
Pelargonidin	0.118
Cyanidin 3-arabinoside	0.914 **
Cyanidin 3-(6''-succinyl-glucoside)	−0.556 *
Peonidin-3-glucoside	−0.554 *
Cyanidin 3-sambubioside 5-glucoside	0.736 **
Cyanidin-3-O-(6''-O-malonyl-2''-O-glucuronyl) glucoside	0.102
Petunidin 3-O-glucoside	−0.162
Malvidin 3-glucoside	0.783 **
Malvidin 3,5-diglucoside	0.509 *
Delphinidin	−0.635 **
3-O-(6''-O-malonyl)-beta-D-glucoside	−0.743 **
Delphinidin 3-O-3'',6''-O-dimalonylglucoside	−0.488 *
Delphinidin 3,5-di(6-O-malonylglucoside)	−0.488 *

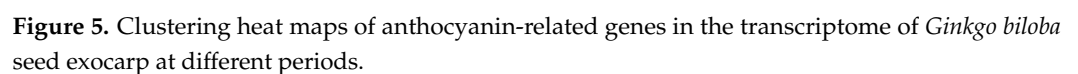
Note: ** Significant correlation at 0.01 level; * Significant correlation at 0.05 level.

During the color change of *Ginkgo biloba* seed exocarp, the content of anthocyanins showed an increasing trend, and three kinds of anthocyanins with increasing content were identified as the key anthocyanins, among which Cyanidin 3-arabinoside has an important effect on the color change of the seed exocarp. The content of Delphinidin 3-O-3'',6''-O-dimalonylglucoside showed a decreasing trend, which suggests that it might be involved in the formation of the color change of *Ginkgo biloba* seed exocarp together with the other three anthocyanins with increasing content (Figure 4).

**Figure 4.** Heat map of four key anthocyanins in different developmental stages of *Ginkgo biloba* seed exocarp. Cy and Mv are cyanidin and malvidin, and Dp is delphinidin.

2.4. Screening of Anthocyanin-Related Genes in the Transcriptome of *Ginkgo biloba* Seed Exocarp at Three Different Color Periods

A total of 95 anthocyanin-related genes, including 42 structural genes and 53 transcription factors, were screened from the transcriptome data of the *Ginkgo biloba* seed exocarp at three periods. In terms of structural genes, six *CHSs*, one *CHI*, two *F3Hs*, two *F3'Hs*, one *F3'5'H*, five *DFRs*, six *ANSs*, eight *UFGTs*, five *SCPL-ATs*, four *BAHD-ATs* and two *OMTs* were screened. In terms of transcription factors, 12 *MYBs*, 7 *bHLHs*, 6 *WDRs*, 8 *AP2/ERFs*, 2 *MADS-boxs*, 7 *b-ZIPs*, 7 *NACs* and 4 *WRKYs* were screened. Among these genes, there were 57 genes with a differential expression tendency (28 structural genes and 29 transcription factors), among which 16 genes showed an upregulated expression tendency and 41 genes showed a downregulated expression tendency (Figure 5).



We applied the Pearson correlation coefficient (PCC) to the analysis. The positive metabolites Cyanidin 3-arabinoside, Malvidin 3-glucoside, Cyanidin 3-sambubioside 5-glucoside and negative metabolites Delphinidin 3-O-3'',6''-O-dimalonylglucoside were correlated with differentially expressed genes from transcriptomic screening to obtain candidate structural genes and transcription factors for seed exocarp color formation (Tables 4–8).

Table 4. Correlation analysis between four key anthocyanins and differentially expressed structural genes in the *Ginkgo biloba* seed exocarp.

Gene Category	Anthocyanin		Cyanidin 3-Arabinoside	Cyanidin 3-Sambubioside 5-Glucoside	Malvidin 3-O-Glucoside	Delphinidin 3-O-3'',6''-O-Dimalonylglucoside
	Gene_ID					
CHS	Gb_01519		−0.9454 **	−0.8984 **	−0.8650 **	0.7533 *
	Gb_19001		−0.7933 *	−0.8832 **	−0.6161	0.8927 **
	Gb_19005		−0.7489 *	−0.8642 **	−0.5462	0.9376 **
	Gb_20355		−0.9414 **	−0.8303 **	−0.8707 **	0.6744 *
	Gb_35771		−0.7504 *	−0.8693 **	−0.5488	0.9314 **
CHI	Gb_07234		−0.8739 **	−0.8606 **	−0.7040 *	0.9352 **
	DFR	Gb_22280	0.8948 **	0.7334 *	0.9368 **	−0.4432
		Gb_09086	−0.7420 *	−0.8550 **	−0.5661	0.9060 **
		Gb_17915	−0.9338 **	−0.8243 **	−0.9139 **	0.6163
		Gb_26459	−0.7812 *	−0.8777 **	−0.5852	0.9231 **
ANS		Gb_26470	−0.8823 **	−0.9206 **	−0.7377 *	0.8827 **
		Gb_21859	−0.7498 *	−0.8596 **	−0.5288	0.9764 **
		Gb_21868	−0.7308 *	−0.8559 **	−0.5271	0.9408 **
		Gb_21869	−0.7421 *	−0.8644 **	−0.5401	0.9268 **
		Gb_21870	−0.7367 *	−0.8581 **	−0.5281	0.9510 **
UFGT		Gb_33402	0.9217 **	0.7555 *	0.9519 **	−0.4899
		Gb_14885	0.9144 **	0.7710 *	0.9321 **	−0.5035
		Gb_35187	0.9174 **	0.8260 **	0.9217 **	−0.5417
		Gb_40295	0.8296 **	0.8995 **	0.6740 *	−0.8719 **

Note: ** Significant correlation at 0.01 level; * Significant correlation at 0.05 level.

Table 5. Correlation analysis between anthocyanins and differentially expressed *F3'H* gene in the dihydroquercetin metabolism pathway of *Ginkgo biloba* seed exocarp.

Gene Category	Anthocyanin		Cyanidin 3-Arabinoside	Cyanidin 3-Sambubioside 5-Glucoside
	Gene_ID			
<i>F3'H</i>	<i>Gb_19800</i>		−0.9343 **	−0.9054 **

Note: ** Significant correlation at 0.01 level.

Table 6. Correlation analysis between anthocyanins and differentially expressed *F3'5'H* gene in the dihydromyricetin metabolism pathway of *Ginkgo biloba* seed exocarp.

Gene Category	Anthocyanin		Malvidin 3-O-Glucoside	Delphinidin 3-O-3'',6''-O-Dimalonylglucoside
	Gene_ID			
<i>F3'5'H</i>	<i>Gb_10102</i>		−0.5153	0.8876 **

Note: ** Significant correlation at 0.01 level.

Table 7. Correlation analysis between Delphinidin 3-O-3'',6''-O-dimalonylglucoside and differential expression of acyltransferase in *Ginkgo biloba* seed exocarp.

Gene Category	Anthocyanin		Delphinidin 3-O-3'',6''-O-Dimalonylglucoside
	Gene_ID		
SCPL-AT	Gb_00744		0.7883 *
	Gb_00876		0.9746 **
	Gb_29794		0.9563 **
	Gb_32811		0.5962
BAHD-AT	Gb_04657		−0.4095
	Gb_21450		−0.5352
	Gb_13282		0.9730 **

Note: ** Significant correlation at 0.01 level; * Significant correlation at 0.05 level.

Table 8. Correlation analysis of four key anthocyanins and differentially expressed transcription factors in *Ginkgo biloba* seed exocarp.

Gene Category	Anthocyanin Gene_ID	Cyanidin 3-Arabinoside	Cyanidin 3-Sambubioside 5-Glucoside	Malvidin 3-O-Glucoside	Delphinidin 3-O-3'',6''-O-Dimalonylglucoside
R2R3MYB	Gb_33428	0.9344 **	0.8886 **	0.8570 **	−0.7317 *
	Gb_05115	−0.9410 **	−0.9000 **	−0.8146 **	0.8692 **
	Gb_18153	−0.9411 **	−0.8928 **	−0.8418 **	0.8202 **
	Gb_19348	−0.8389 **	−0.7203 *	−0.7460 *	0.6809 *
	Gb_24073	−0.7473 *	−0.8339 **	−0.5216	0.9309 **
	Gb_24641	−0.7752 *	−0.8657 **	−0.5611	0.9777 **
	Gb_38090	−0.7171 *	−0.7002 *	−0.4960	0.9016 **
	Gb_39484	−0.9234 **	−0.7769 *	−0.9480 **	0.4516
R3MYB	Gb_39852	−0.8228 **	−0.8621 **	−0.7127 *	0.8204 **
	Gb_15334	−0.8798 **	−0.9232 **	−0.7282 *	0.9159 **
bHLH	Gb_34778	0.9096 **	0.7898 *	0.9647 **	−0.4727
	Gb_14057	−0.9162 **	−0.8361 **	−0.9029 **	0.5916
	Gb_21995	−0.8852 **	−0.7791 *	−0.7970 *	0.7345 *
	Gb_33185	−0.8234 **	−0.8723 **	−0.6158	0.9653 **
WDR	Gb_37351	0.9052 **	0.7658 *	0.7915 *	−0.7554 *
	Gb_40146	0.8264 **	0.7905 *	0.8317 **	−0.5701
	Gb_30748	−0.8180 **	−0.7298 *	−0.9117 **	0.3287
b-ZIP	Gb_12023	0.8683 **	0.9037 **	0.7016 *	−0.8585 **
	Gb_07087	−0.9076 **	−0.8475 **	−0.8071 **	0.8107 **
	Gb_41342	−0.7924 *	−0.7049 *	−0.8346 **	0.4522
NAC	Gb_05670	0.8730 **	0.7530 *	0.9317 **	−0.4294
	Gb_13930	0.8682 **	0.7361 *	0.9382 **	−0.4134
	Gb_17882	0.9013 **	0.9164 **	0.7601 *	−0.8778 **
	Gb_18916	0.8913 **	0.8834 **	0.8043 **	−0.7130 *
AP2/ERF	Gb_21446	−0.9266 **	−0.7874 *	−0.8803 **	0.7077 *
	Gb_07474	−0.8226 **	−0.7134 *	−0.6785 *	0.8140 **
	Gb_26662	−0.8952 **	−0.7758 *	−0.7770 *	0.7500 *
MADS-box	Gb_41549	−0.9357 **	−0.8845 **	−0.8765 **	0.7646 *
	MSTRG.2320	−0.9227 **	−0.8616 **	−0.8772 **	0.7407 *

Note: ** Significant correlation at 0.01 level; * Significant correlation at 0.05 level.

According to the anthocyanin-related structural genes screened from the transcriptome of *Ginkgo biloba* seed exocarp, we constructed the anthocyanin-related accumulation process during the transcolor process of *Ginkgo biloba* seed exocarp (Figure 6). Among the structural genes, we found that in *GbANS*, only *Gb_33402* showed an upregulated expression trend: the expression level of the *Ginkgo biloba* seed exocarp in the orange-red period was 3.08 times that in the yellow period, and the expression level in the yellow period was 1.05 times that in the green period, and the correlation with Cyanidin 3-arabinoside, Malvidin 3-glucoside and Cyanidin 3-sambubioside 5-glucoside reached 0.9217, 0.9519 and 0.7555, respectively. It showed the highest correlation with Cyanidin 3-arabinoside, the most critical anthocyanin for color formation in *Ginkgo biloba* seed exocarp, so it is an important structural gene for anthocyanin accumulation, and may be the key structural gene involved in the color formation of *Ginkgo biloba* seed exocarp. In addition, one *GbDFR* gene (*Gb_22280*) and two *GbUGFT* genes (*Gb_14885* and *Gb_35187*) were screened, and they were strongly correlated with Cyanidin 3-arabinoside, Malvidin 3-glucoside and Cyanidin 3-sambubioside 5-glucoside ($PCC > 0.7$), and *Gb_22280* was highly expressed in the orange-red stage of the *Ginkgo biloba* seed exocarp. *Gb_14885* and *Gb_35187* were highly expressed in the orange-red stage and yellow stage, and the expression of the orange-red stage was 12.33 times and 6.16 times, respectively, compared with the yellow stage. Most of the structural genes in the upstream pathway of anthocyanin synthesis were positively correlated with Delphinidin 3-O-3'',6''-O-dimalonylglucoside, among which one *GbANS* (*Gb_21859*) showed the highest correlation, and the PCC reached −0.9764. It

was only expressed in large quantities in the green stage of the *Ginkgo biloba* seed exocarp. Secondly, we also screened two acyltransferase genes, *GbSCPL* (*Gb_00876*) and *GbBAHD* (*Gb_13282*), which were strongly related to Delphinidin 3-O-3'',6''-O-dimalonylglucoside. The PCC between them and Delphinidin 3-O-3'',6''-O-dimalonylglucoside reached -0.9746 and -0.9730 , respectively, and they were only highly expressed in the green phase of the exocarp. Almost no expression was found in the yellow and orange-red periods (Tables 4–7, Figure 7B,C).

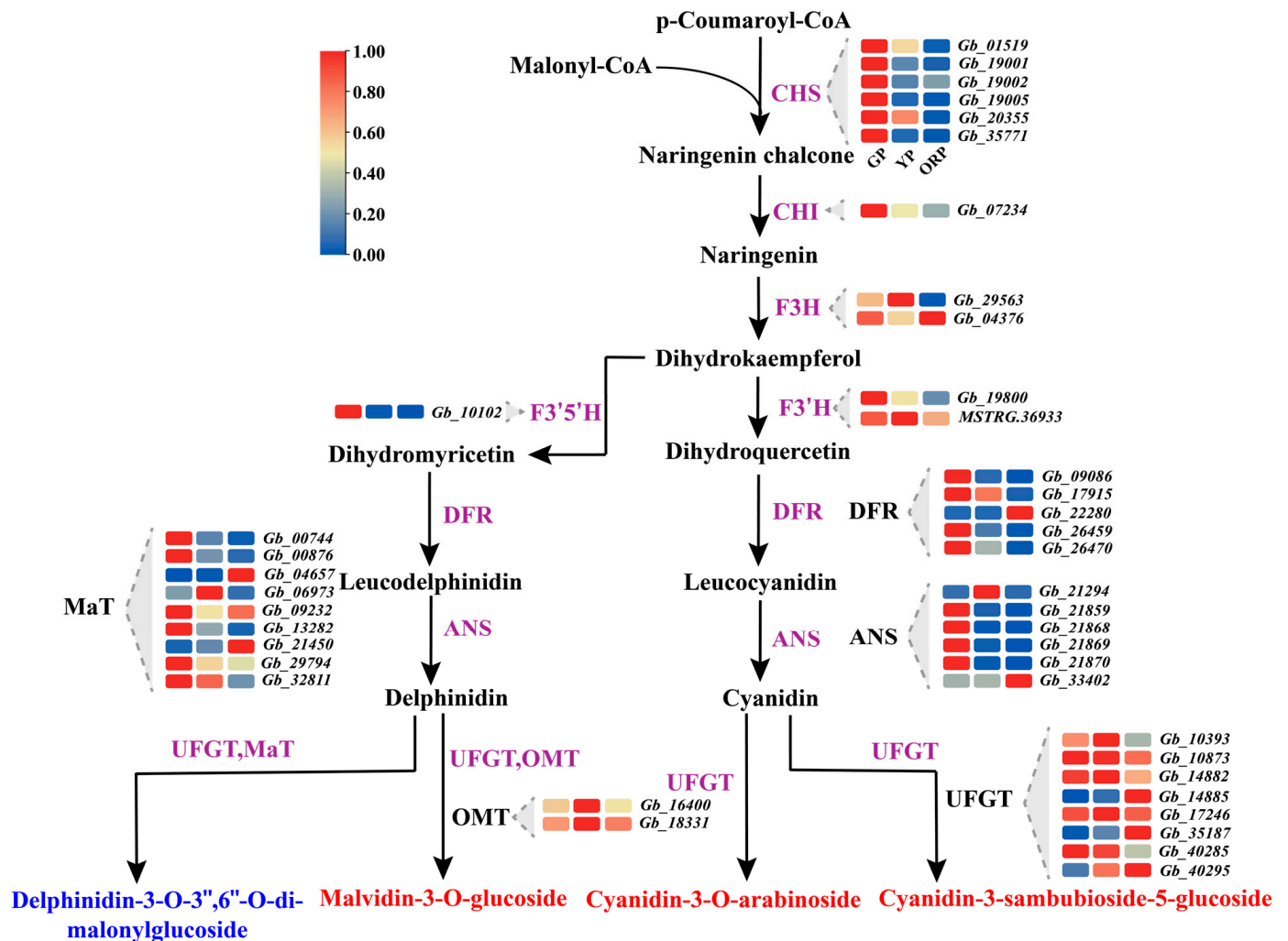


Figure 6. Anthocyanin metabolic pathway of *ginkgo biloba* seed exocarp. Overview of anthocyanin biosynthesis pathways and temporal expression patterns of related proteins in *Ginkgo biloba* seed exocarp. *CHS*, chalcone synthetase; *CHI*, chalcone isomerase; *F3H*: flavanone-3-hydroxylase; *F3'H*: flavonoid 3'-hydroxylase; *F3'5'H*: flavonoid-3',5'-hydroxylase; *DFR*: dihydroflavonol-4-reductase; *ANS*, anthocyanin synthetase; *UFGT*: flavonoid glucosyltransferase; *OMT*, methyltransferase; *MaT*: malonyltransferase.

Among the transcription factors, we found that only *Gb_33428* was positively correlated with Cyanidin 3-arabinside, Malvidin 3-glucoside and Cyanidin 3-sambubioside 5-glucoside among all *MYB* transcription factors. The PCC reached 0.9344, 0.8570 and 0.8886, respectively. We also screened one *bHLH* (*Gb_34778*) and one *WDR* (*Gb_37351*), which showed a strong correlation with these three anthocyanins. The PCCs reached 0.9096, 0.9647 and 0.7898; and 0.9052, 0.7915 and 0.7658, respectively. In addition, one *b-ZIP* (*Gb_12023*) and four *NACs* (*Gb_05670*, *Gb_13930*, *Gb_17882* and *Gb_18916*) had a significant positive correlation with these three anthocyanins (PCC > 0.7). Six *R2R3MYBs*

(*Gb_05115*, *Gb_18153*, *Gb_24073*, *Gb_24641*, *Gb_38090* and *Gb_39852*) and one *R3MYB* (*Gb_15334*) with Delphinidin 3-O-3'',6''-O-dimalonylglucoside showed a strong positive correlation (PCC > 0.8), in which *Gb_24641* had the highest correlation with this negative anthocyanin, and the PCC reached −0.9777. One *bHLH* (*Gb_33185*) was also screened, and the PCC of this negative metabolism anthocyanin reached −0.9653. In addition, one *b-ZIP* (*Gb_07087*) and one *AP2/ERF* (*Gb_07474*) were also identified as strongly correlated with this negative metabolism of anthocyanins (PCC > 0.8) (Table 8, Figure 7B,C).

We also conducted a co-expression correlation analysis of seven structural genes that were screened and strongly correlated with four key anthocyanins and eighteen transcription factors (Table 9). Among the *MYB* transcription factors, only *Gb_33428* was positively correlated with *Gb_33402*, a key enzyme for anthocyanin synthesis in seed exocarp (PCC = 0.9006). All the other *MYB* transcription factors were negatively correlated with *Gb_33402*, and *Gb_18153* had the highest negative correlation with *Gb_33402* (PCC = −0.8624). The positive correlation between one *bHLH* (*Gb_34778*) and two *NACs* (*Gb_05670* and *Gb_13930*) and *Gb_33402* was also more than 0.9, indicating that they may regulate and affect the formation of the seed exocarp color of *Ginkgo biloba* through the positive regulation of *Gb_33402*.

Table 9. Correlation analysis between 18 key transcription factors and 7 key structural genes in *Ginkgo biloba* seed exocarp.

Gene Category	Structure Gene	<i>Gb_33402</i> (ANS)	<i>Gb_22280</i> (DFR)	<i>Gb_21859</i> (ANS)	<i>Gb_14885</i> (UFGT)	<i>Gb_35187</i> (UFGT)	<i>Gb_00876</i> (SCPL-AT)	<i>Gb_13282</i> (BAHD-AT)
<i>R2R3MYB</i>	<i>Gb_33428</i>	0.9006 **	0.8951 **	−0.8135 **	0.9265 **	0.9434 **	−0.8481 **	−0.8521 **
	<i>Gb_05115</i>	−0.8540 **	−0.8199 **	0.8795 **	−0.8552 **	−0.8715 **	0.9197 **	0.9539 **
	<i>Gb_18153</i>	−0.8624 **	−0.8401 **	0.8730 **	−0.8759 **	−0.8934 **	0.9020 **	0.9236 **
	<i>Gb_24073</i>	−0.5431	−0.5242	0.9581 **	−0.5800	−0.6157	0.9656 **	0.9145 **
	<i>Gb_24641</i>	−0.5793	−0.5462	0.9983 **	−0.6058	−0.6465	0.9959 **	0.9786 **
	<i>Gb_38090</i>	−0.5076	−0.4672	0.8544 **	−0.5107	−0.5204	0.8848 **	0.8789 **
	<i>Gb_39852</i>	−0.7271 *	−0.7008 *	0.8811 **	−0.7464 *	−0.7825 *	0.8818 **	0.8996 **
<i>R3MYB</i>	<i>Gb_15334</i>	−0.7411 *	−0.7112 *	0.9565 **	−0.7594 *	−0.7950 *	0.9678 **	0.9713 **
<i>bHLH</i>	<i>Gb_34778</i>	0.9765 **	0.9789 **	−0.5496	0.9755 **	0.9765 **	−0.6107	−0.6491
	<i>Gb_33185</i>	−0.6451	−0.6155	0.9786 **	−0.6687 *	−0.6995 *	0.9923 **	0.9773 **
<i>WDR</i>	<i>Gb_37351</i>	0.8520 **	0.8367 **	−0.7892 *	0.8683 **	0.8591 **	−0.8435 **	−0.8580 **
<i>b-ZIP</i>	<i>Gb_12023</i>	0.7350 *	0.7176 *	−0.9292 **	0.7699 *	0.8037 **	−0.9385 **	−0.9164 **
	<i>Gb_07087</i>	−0.8532 **	−0.8426 **	0.8440 **	−0.8706 **	−0.8783 **	0.8887 **	0.9006 **
<i>NAC</i>	<i>Gb_05670</i>	0.9414 **	0.9482 **	−0.5347	0.9510 **	0.9524 **	−0.5888	−0.6123
	<i>Gb_13930</i>	0.9394 **	0.9396 **	−0.5114	0.9406 **	0.9410 **	−0.5632	−0.6031
	<i>Gb_17882</i>	0.7941 *	0.7724 *	−0.9309 **	0.8182 **	0.8472 **	−0.9501 **	−0.9507 **
	<i>Gb_18916</i>	0.8635 **	0.8599 **	−0.8040 **	0.8960 **	0.9208 **	−0.8297 **	−0.8270 **
<i>AP2/ERF</i>	<i>Gb_07474</i>	−0.7397 *	−0.7125 *	0.7788 *	−0.7387 *	−0.7299 *	0.8331 **	0.8669 **

Note: ** Significant correlation at 0.01 level; * Significant correlation at 0.05 level.

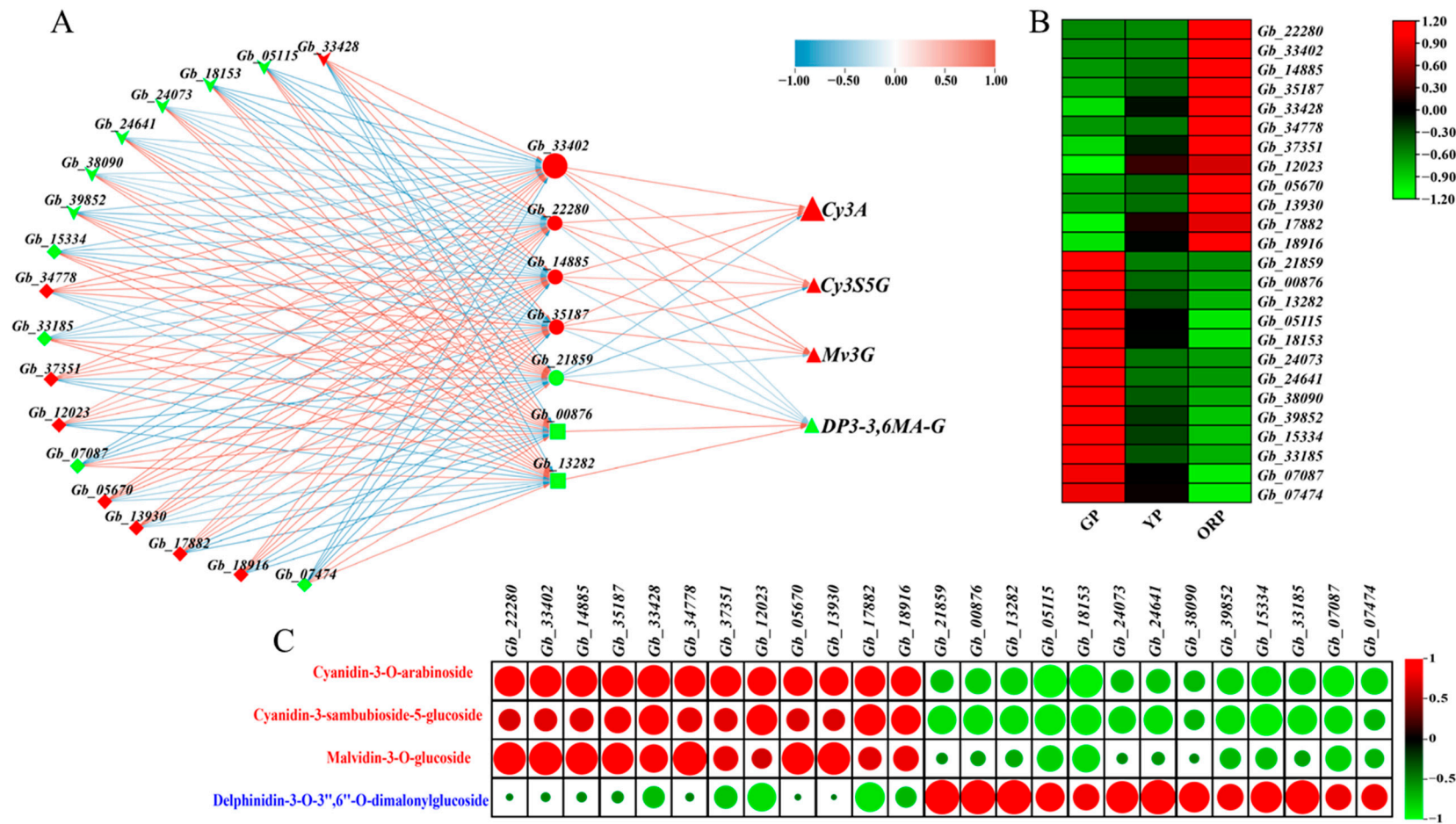


Figure 7. Correlation analysis of 4 key anthocyanins in *Ginkgo biloba* seed exocarp with key structural genes and transcription factors. (A) Vector map of anthocyanins and key gene regulatory networks in *Ginkgo biloba* exocarp. Triangle: four key anthocyanins Cy3A, Cy3S5G, Mv3G, DP3-3,6MA-G are Cyanidin-3-O-arabinoxide, Cyanidin-3-sambubioside-5-glucoside, Malvidin-3-O-glucoside and Delphinidin-3-O-3'',6''-O-dimalonylglucoside; Square: acyltransferase on the dihydromyricetin shard; Round: Other structural genes of anthocyanin metabolic pathway; V type: R2R3MYB; Diamond: other transcription factors; Red: Increased content or expression; Green: Decreased content or expression. (B) Expression heat maps of 25 key genes in three periods of *Ginkgo biloba* exocarp. (C) Heat map of correlation between four anthocyanins in *Ginkgo biloba* exocarp and 25 key genes.

Finally, we constructed the co-expression network of the selected key structural genes and transcription factors and four key anthocyanins (Figure 7A).

2.6. qRT-PCR Verification of Differentially Expressed Genes in *Ginkgo biloba* Seed Exocarp Transcriptome Data

In order to further verify the reliability and accuracy of the transcriptome data, seven key structural genes, key MYB transcription factors and other differentially expressed transcription factors (a total of twenty key candidate genes were selected) were screened for qRT-PCR verification, and their expression levels (GP, YP and ORP) in different periods of *Ginkgo biloba* seed exocarp were detected. The relative expression trends of these candidate genes were similar to those of RNA-seq, indicating the high reliability and accuracy of the transcriptome data (Figure 8).

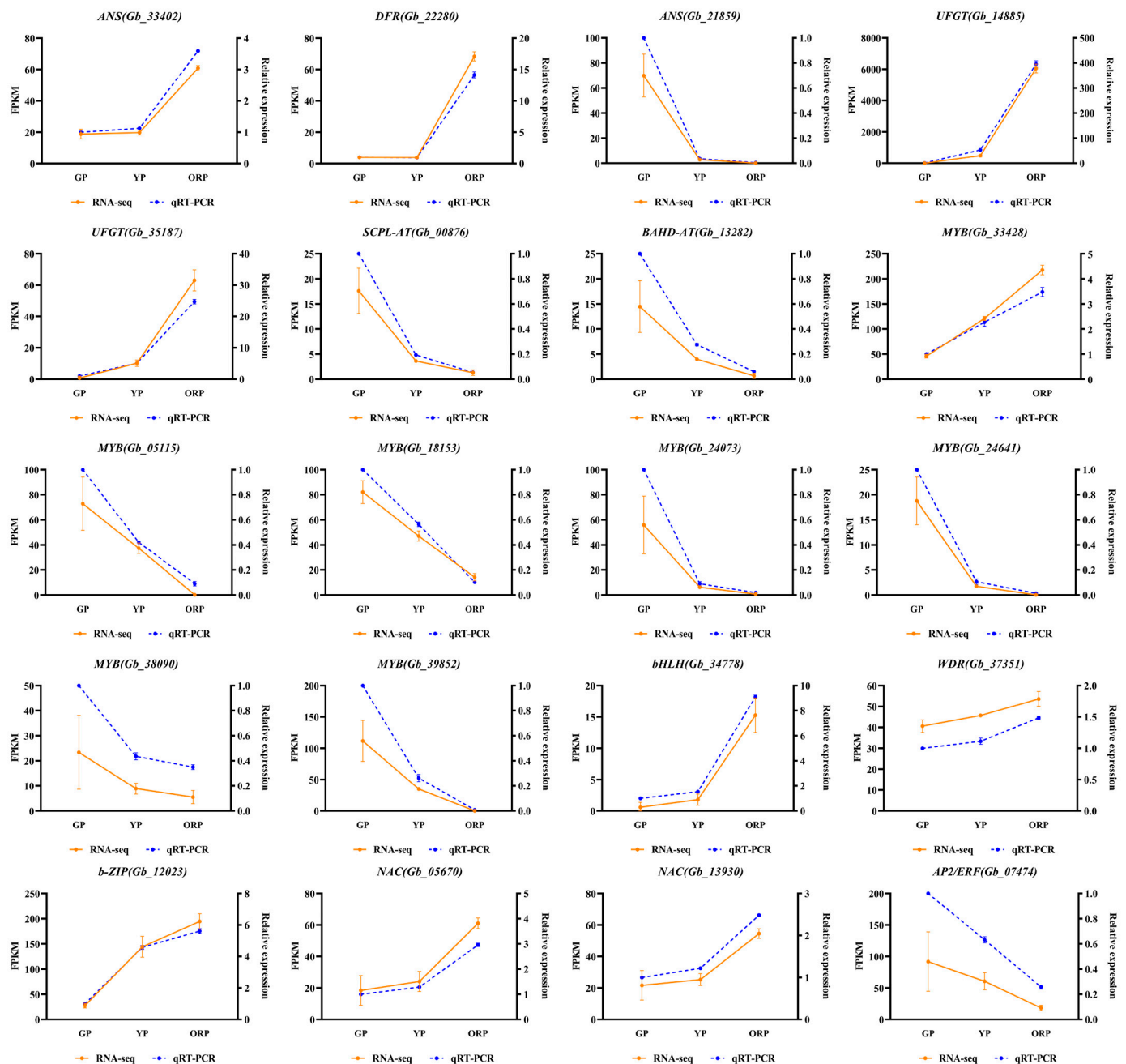


Figure 8. Detection of 20 key genes in *Ginkgo biloba* seed exocarp by qRT-PCR.

3. Discussion

This study determined that the redness (a^*) value was closely related to the formation of the seed exocarp color through the determination of color parameters in different color periods. During the process of *Pistacia chinensis* leaves turning red, the anthocyanin content of the leaves showed an increasing trend, and the anthocyanin was significantly positively correlated with a^* [36], which was consistent with the process of the color change in the seed exocarp of *Ginkgo biloba* in this study. Through the correlation analysis of color parameters, it was found that there was an extremely significant positive correlation between the lightness (L^*) and the yellowness (b^*) of *Ginkgo biloba* seed exocarp, and it was determined that the cell lightness presented by different colors of *Ginkgo biloba* seed exocarp was mainly affected by the yellowness, which was consistent with the research results of Li [37] in *Bauhinia purpurea* petals.

In this study, Cyanidin 3-arabinoside, Cyanidin 3-sambubioside 5-glucoside, Malvidin 3-glucoside and Delphinidin 3-O-3'',6''-O-dimalonylglucoside were the main anthocyanins that caused the color change in *Ginkgo biloba* seed exocarp. Relevant studies have shown that in *Acer triflorum* Komarov, Cyanidin 3-arabinoside is significantly increased in red and orange leaves and is a key anthocyanin in leaf discoloration [13]. During the process of red *Ziziphus jujuba* Mill, the content of Malvidin 3-glucoside increases, which plays a key role [38]. When the leaves of *Padus virginiana* change from green to purplish red, Malvidin 3-glucoside is significantly correlated with leaf color [14]. This is consistent with the yellow and orange-red color of the seed exocarp in *Ginkgo biloba*; the difference is that there are two kinds of anthocyanins in *Ginkgo biloba* seed exocarp, which are Cyanidin 3-sambubioside 5-glucoside and Delphinidin 3-O-3'',6''-O-dimalonylglucoside, respectively, and they may work together to influence the color change of the seed exocarp. There is no research reporting that these two kinds of anthocyanins may play a role in the development of red or yellow colors in plants.

In the process of anthocyanin synthesis, many structural genes and transcription factors play a crucial role. Previous studies have shown that many structural genes are involved in the formation of red color in plants [39]. In this study, one *ANS* (*Gb33402*) plays a key role. It had the highest correlation with Cyanidin 3-arabinoside (PCC = 0.9217), and in *Acer triflorum* Komarov, while Cyanidin 3-arabinoside increased significantly in red and orange leaves, the expression of *ANS* (*Cluster-24474.33469*) also showed an upregulated trend [13]. In addition, when the green skin of *Zanthoxylum bungeanum* Maxim turned red during the development of *Zanthoxylum*, the transcriptome data analysis showed that *ANSs* (*c158341*, *c99788*, *c97481*, *c125833*) could promote the accumulation of cyanidin-o-syringic acid in the peel of *Zanthoxylum*, causing the fruit to change from green to red [16]. In addition, brightly colored fruits are often characterized by the high gene expression of the key downstream enzymes of the anthocyanin biosynthesis pathway, such as the enzymes encoding *DFR*, *ANS*, and *UFGT* [40]. The key gene *GbDFR* (*Gb_22280*) and two *GbUFGT* genes (*Gb_14885* and *Gb_35187*) in the middle and lower reaches of the exoderm were also highly expressed during the orange-red period. The candidate key transcription factors (*MYB*, *bHLH*, *b-ZIP*, *NAC*, *WDR* and *AP2/ERF*) screened in *Ginkgo biloba* seed exocarp have been reported in other studies to be involved in regulating the formation of red color in plants [41–43].

4. Materials and Methods

4.1. Plant Materials

The *Ginkgo biloba* seeds were collected from a female *Ginkgo biloba* tree (Zhongnanlin No. 2) growing in the botanical garden of the Central South University of Forestry and Technology (28°8' N, 112°59' E). The samples were collected every month since 15 May 2023 and divided into three periods according to the color and phenotype of the seed exocarp. The periods were as follows: 15 May–15 June was the green period (GP), 15 July–15 August was the yellow period (YP), and 15 September–15 October was the orange-red period (ORP). During the sampling, the middle growth seeds of the female plants with good growth were

collected, washed with sterile distilled water after collection and dried. After separating the seed exocarp from the seed kernels, the obtained seed exocarp were quickly frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ for later use.

4.2. Determination of Color Parameters and Anthocyanin Content of *Ginkgo biloba* Seed Exocarp

Ginkgo biloba seed exocarp at different development stages were wiped clean, and the middle position of *Ginkgo biloba* seed exocarp was measured with a 3nh spectrophotometer (YS2580) under the conditions of a D65 light source and 10° observation angle [44]. Three *Ginkgo biloba* seed exocarp in each period were measured, and each *Ginkgo biloba* seed was measured three times, and the average value was taken, and the L^* , a^* and b^* values were recorded after measurement. For the determination of anthocyanin content [45], take healthy *ginkgo biloba* seeds, wipe the surface of the *Ginkgo biloba* seed exocarp, weigh 1.5 g of *Ginkgo biloba* seed exocarp and cut it, put it in a 10 mL centrifuge tube, add 0.1% HCL-methanol solution and fill it to 5 mL, seal it with a sealing film, and soak it in a refrigerator at $4\text{ }^{\circ}\text{C}$ for 24 h away from light. This was repeated for each sample three times. After leaching, the absorbance of the extract was measured at the wavelength of 530 nm, and the anthocyanin content was calculated. The relative content of anthocyanin (mg/g) = $\text{OD}_{530}/\text{g} \times \text{FW}$.

4.3. Metabolome and Transcriptome Data Materials

The metabolome and transcriptome data of *Ginkgo biloba* seed exocarp were obtained from previous laboratory work. The samples were collected from a female tree (Zhongnanlin No. 2) located in the campus of Central South University of Forestry and Technology (E: $112^{\circ}59'$; N: $28^{\circ}8'$; altitude: 94 m, Changsha, China). Twenty *Ginkgo biloba* seeds for each sample were collected at 60 d (15 June, the green period), 90 d (1 August, the yellow period) and 120 d (15 September, the orange-red period) after pollination, and the samples were named GP, YP and ORP. Three biological replicates were collected for each sample.

4.4. Real-Time Fluorescence Quantitative PCR Verification

A total of 20 differentially expressed genes were verified by qRT-PCR. Total RNA was extracted using an Omega RNA extraction kit (OMEGA, Norcross, GA, USA). cDNA was obtained by reverse transcription according to a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The qRT-PCR program was carried out for each sample using a SYBR Green Premix Pro Taq HS qPCR Kit (High Rox Plus) (Accurate Biotechnology (Hunan province, China) Co., Ltd., Changsha, China) with an ABI Step One Plus real-time PCR system, and the parameters were set as follows: stage 1, $95\text{ }^{\circ}\text{C}$ for 30 s; stage 2, 40 cycles of $95\text{ }^{\circ}\text{C}$ for 5 s and $60\text{ }^{\circ}\text{C}$ for 30 s; and stage 3, melting curve. The *GAPDH* gene was used as the internal reference gene [46]. Three biological replicates with internal repeats were set for each sample. We used the online Oligo Architect™ fluorescence quantitative primer (<http://www.oligoarchitect.com/Login.jsp>) design (accessed on 13 December 2023). The specific primers of the anthocyanin biosynthesis-related genes and *GAPDH* genes are listed in Table 10. The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [47].

Table 10. Specific primers for anthocyanin biosynthesis-related genes and *GAPDH*.

Primer Name	Sequence (5'→3')
<i>GAPDH</i> -F	CAAGGACTCCAACACCTTACTC
<i>GAPDH</i> -R	CCGTGGATTCAACCACATACT
<i>Gb_33402</i> -F	CCTGGTGCTCTGATTGTGAATATCG
<i>Gb_33402</i> -R	TGACATCCTTGATTGGTCCTTATGC
<i>Gb_22280</i> -F	CGAATAGCGGCACAGAGCAGAA
<i>Gb_22280</i> -R	TGACAGTAGCATGGACGTTGTAACC
<i>Gb_21859</i> -F	TGGTGCCTGGTCTCCAACCTCTT

Table 10. Cont.

Primer Name	Sequence (5'→3')
<i>Gb_21859</i> -R	AGCCCACTCTTGATTTGCCATTGC
<i>Gb_14885</i> -F	GGCGTTCCAATGCTCAGTGTTT
<i>Gb_14885</i> -R	CGCTTATTCAGTCGCAATCCAGTCT
<i>Gb_35187</i> -F	CCATTAGAGACGCAGAAGGAGAGT
<i>Gb_35187</i> -R	AATGTGAACGGTTCGAGCATAA
<i>Gb_00876</i> -F	TCTAAGCCTCTGGTTCTGTGGTTGA
<i>Gb_00876</i> -R	GAGCGACTTTCATCCGAGTTGAC
<i>Gb_13282</i> -F	TCCGCCCTTATCTCCGTCCTTTCTTT
<i>Gb_13282</i> -R	TGCTCCATACCACATTCGGTCACT
<i>Gb_33428</i> -F	GCAGCAATCTGGAGCCGAAGG
<i>Gb_33428</i> -R	CCGTCGCCCTTGACTCTCATTTC
<i>Gb_05115</i> -F	CCGTGCTGCGAGAAGGTTGG
<i>Gb_05115</i> -R	GCTCTTTCCACACCGTAACAGACC
<i>Gb_18153</i> -F	TAGCATAAGCAGAGCCACCACAG
<i>Gb_18153</i> -R	ACAGCAGCCTCCTCCATTGATT
<i>Gb_24073</i> -F	ACAGCGGTTAAGATGGCGGTTG
<i>Gb_24073</i> -R	TGCGATTGGAATCCTGCGGTTG
<i>Gb_24641</i> -F	ATCCATTGCGTGTTCCCGACTAG
<i>Gb_24641</i> -R	GCTTCCGCTCGCTATCTCATCAG
<i>Gb_38090</i> -F	CCGTCCCCATTAATCCACCAAC
<i>Gb_38090</i> -R	GCTGTGCCTCCTTCATGCTGTC
<i>Gb_39852</i> -F	GCTGCTTGGATACAGATCAGGATG
<i>Gb_39852</i> -R	CCGCCGAGAATTTGGGAGAG
<i>Gb_34778</i> -F	TCTGGGTGTCGGAAGCGGATAA
<i>Gb_34778</i> -R	CTGAGCCTCCACGAAATCCACTT
<i>Gb_37351</i> -F	GCTTCAACCTTGGCAGTGTCATC
<i>Gb_37351</i> -R	GCTTCCTCTTCTTCATCACGCTCTA
<i>Gb_12023</i> -F	ATCATTGACGGCTTTGGCGGTAG
<i>Gb_12023</i> -R	GATTGGTGGCAGGATAGGCGATA
<i>Gb_05670</i> -F	AAGAAACTGTTACTCCAGCCGAAGA
<i>Gb_05670</i> -R	AGCAGCCTGTAGACCAATTCATT
<i>Gb_13930</i> -F	TCCACCAGGTCAAGAGATCCAATCC
<i>Gb_13930</i> -R	GTATGGCGGCATCCAATGTCTGT
<i>Gb_07474</i> -F	CGCCCATCACTCAGCATTTC
<i>Gb_07474</i> -R	AGCCTCCTTCAGAACGCCATAA

4.5. Statistical Analysis

Each measurement was performed in triplicate. SPSS 26.0 (IBM, New York, NY, USA) was used for significance and correlation analyses; $p < 0.05$ was considered statistically significant. Line graphs were plotted using GraphPad Prism 9.0 (GRAPHPAD SOFTWARE, LLC., Boston, CA, USA), and the results represent the mean $\pm$ SD of data from three independent experiments. Heat mapping was created using TBtools (South China Agricultural University, Guangdong province, China) and visualization of the corresponding relevant network analysis using Cytoscape software (version 3.7.0). Adobe Illustrator (Adobe Inc., Mountain View, CA, USA) was used to process and beautify the images.

5. Conclusions

In the process of the color change of the *Ginkgo biloba* seed exocarp, the anthocyanin content showed an accumulation trend, and the color parameter a^* was closely related to the color change of the seed exocarp. We used the combined analysis of transcriptomes and metabolomes to show that Cyanidin 3-arabinoside has a great influence on the color change of the seed exocarp. Malvidin 3-glucoside, Cyanidin 3-sambubioside 5-glucoside and Delphinidin 3-O-3'',6''-O-dimalonylglucoside may jointly affect the formation of seed exocarp color. A total of 7 structural genes and 18 transcription factors were identified in the transcriptome, among which one *ANS* (*Gb_33402*) had the highest correlation with

Cyanidin 3-arabinoxide (PCC = 0.9217), and suggesting that it may be the key structural gene in the color change of exocarp. Several key structural genes *GbDFR* (*Gb_22280*) and two *GbUGT* genes (*Gb_14885* and *Gb_35187*) were also identified. Among the transcription factors, *MYB*, *bHLH*, *b-ZIP*, *NAC*, *WDR* and *AP2/ERF* may jointly affect the formation of seed exocarp color. The results of this study provide a new understanding of anthocyanin accumulation and color change mechanism in the seed exocarp of *Ginkgo biloba*.

Author Contributions: Conceptualization, J.T., Z.F., M.L. and Y.W.; methodology, J.T., Z.F., M.L. and Y.W.; software, J.T.; validation, J.T. and X.X.; formal analysis, J.T.; investigation, X.X., M.L. and Y.W.; writing—original draft preparation, J.T. and Z.F.; writing—review and editing, J.T., Z.F., M.L. and Y.W.; supervision, M.L. and Y.W.; project administration, Y.W.; funding acquisition, Y.W. All authors have read and agreed to the published version of the manuscript.

Funding: The National Natural Science Foundation of China (32171842) and The National Key Research and Development Program of China (Grant No. 2019YFD1100403).

Data Availability Statement: The transcriptome data of *G. biloba* seed exocarp are deposited as a Bio Project under accession PRJNA1026889.

Conflicts of Interest: The authors declare no conflicts of interest.

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