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Temporal Changes in Flavonoid Components, Free Radical Scavenging Activities and Metabolism-Related Gene Expressions during Fruit Development in Chinese Dwarf Cherry (*Prunus humilis*)

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Abstract: Temporal changes in total flavonoid content (TFC), composition, free radical scavenging activity and metabolism-related gene expression of three *Prunus humilis* cultivars with distinctively different fruit colors were investigated in this study. The highest fruit TFCs of all three cultivars were observed at the initial sampling stage (young-fruit stage, YFS), which then declined gradually until fruit ripening. The dark-red-fruited cultivar 'Jinou 1' had the highest TFC, followed by the yellow-red-fruited cultivar 'Nongda 3' and the yellow-fruited cultivar 'Nongda 5'. Thirteen flavonoid compounds were found in the three cultivars by using high-performance liquid chromatography (HPLC), and the content of most flavonoid compounds gradually decreased throughout the fruit-ripening process, with the exception of cyanidin-3-O-glucoside (C3G). C3G, as the main anthocyanin in *P. humilis* fruits, increased drastically during the fruit-coloring process of cultivars 'Jinou 1' and 'Nongda 3', while it was not detected in the developing fruits of cultivar 'Nongda 5'. The antioxidant activity assay (DPPH, FRAP and ABTS) revealed that fruits of all three cultivars at YFS also had the highest antioxidant activities, and cultivar 'Jinou 1' had the highest antioxidant activities. Correlation analysis revealed that the antioxidant activities were significantly positively correlated with the TFCs and contents of the main compounds such as catechin, proanthocyanidin B1 and phloretin-2′,4-O-diglucoside ($p < 0.01$). Moreover, gene expression analysis showed that the flavonoid biosynthetic genes had different expression patterns in the three cultivars. The expression levels of *ChCHS*, *ChCHI*, *ChF3H*, *ChDFR*, *ChLDOX* and *ChUFGT* increased gradually with fruit ripening in cultivar 'Jinou 1', while all flavonoid-related genes in cultivar 'Nongda 5' decreased gradually during fruit development. The results from our study could significantly contribute to the deeper understanding of flavonoid accumulation mechanisms in *P. humilis* fruits and also help facilitate the targeted cultivar development and the utilization as a functional food of this fruit species.

Keywords: *Prunus humilis*; flavonoids; high-performance liquid chromatography; antioxidant activities

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1. Introduction

Flavonoids are a class of polyphenols, which can be used as effective natural antioxidants to prevent free radical damage in the human body [1]. The steady intake of plant flavonoids reduces the effects of oxidative damage that could lead to many severe illnesses, such as cardiovascular diseases and diabetes [2]. Studies have revealed that fruit flavonoids can have a particularly potent effect on cognition and age-related memory decline in rats [3,4]. Other reports have shown that consuming flavonoid-rich foods, such as apples, pears, berries and peppers, may also help adults maintain body weight and help to prevent potential consequences of obesity [5,6]. In recent years, consumer demand

and interest in the nutraceutical and functional values of plant flavonoids have increased, leading to comprehensive studies on a number of high-flavonoid fruits over the past two decades, such as apple, citrus, blueberry, blackberry, bilberry, raspberry, strawberry and sea buckthorn [7].

Prunus humilis, known as Chinese dwarf cherry or 'Calcium fruit', is a fruit-bearing shrub distributed throughout Northern China [8]. Its native habitat is typically the sunny sides of limestone mountains [9]. Several studies showed that *P. humilis* possesses strong drought tolerance and cold hardiness as well as good adaption to soils with moderate salinity and alkalinity [10]; therefore, *P. humilis* has been used as a key plant species in numerous soil improvement and water conservation projects in Northern China [11]. Besides its ecological value, fruits of *P. humilis* are rich in vitamins, mineral elements, organic acids and other nutrients [12,13]. Recently, the fruits of *P. humilis* were also proved to be flavonoid-rich and a promising source of natural antioxidants [14–17]. The flavonoid biosynthesis pathway in plants is quite conserved, and a number of key enzymes are involved [18,19], such as phenylalanine ammonia lyase (PAL), cinnamic acid 4-carboxylase (C4H), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanol synthase (FLS), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), UDP-glycose flavonoid glycosyltransferase (UGFT), leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR). Although *P. humilis* is flavonoid-rich and has a great potential in the healthcare industry, less is known about the flavonoid biosynthesis in *P. humilis*. Therefore, it is necessary to study the flavonoid accumulation in *P. humilis* extensively to boost breeding of high-flavonoid varieties and develop new health-promoting products.

In the present study, the fruits of three *P. humilis* cultivars, which are widely cultivated and processed in China, were collected at five different developmental stages. The changes in total flavonoid content, flavonoid compositions, free radical scavenging activities and flavonoid biosynthetic gene expressions were investigated in *P. humilis* fruits during their development. Our results can be readily used in the accelerated development and improvement of this commercially valuable species.

2. Materials and Methods

2.1. Plant Materials and Chemicals

Fruits of three *P. humilis* cultivars ('Nongda 3', 'Jinou 1' and 'Nongda 5') were harvested from July to September in the Experimental Garden of Jinzhong University in 2021. Pruning and pest control were conducted according to standard cultivation procedures for each cultivar. The five developmental stages were the young-fruit stage (YFS, 30 d after flowering), pit-hardening stage (PHS, 60 d after flowering), fruit-enlargement stage (FES, 105 d after flowering), color-changing stage (CCS, 120 d after flowering) and fruit-ripening stage (FRS, 130 d after flowering), respectively (Figure 1).

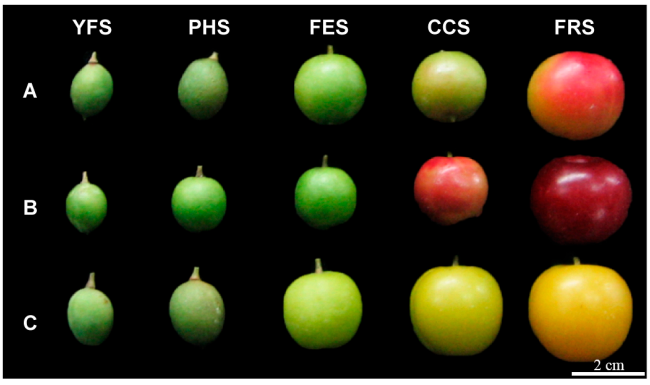

Figure 1. Fruits from three *Prunus humilis* cultivars, 'Nongda 3' (**A**), 'Jinou 1' (**B**) and 'Nongda 5' (**C**), at five different developmental stages: YFS, young-fruit stage; PHS, pit-hardening stage; FES, fruit-enlargement stage; CCS, color-changing stage; and FRS, fruit-ripening stage. Bar = 2.0 cm.

At each developmental stage, 100 fruits from each cultivar (2 per plant) were randomly selected and harvested by hand. After removing the kernel, fruit samples were cut into small pieces and then stored at −80 °C for subsequent experiments. Sampling was repeated thrice.

Methanol (HPLC-grade), water and flavonoid standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and formic acid and acetonitrile were purchased from Alltech Scientific (Beijing, China). The following chemical reagents were acquired from Tianjin Guangfu Fine Chemical Co., Ltd. (Tianjin, China): acetic acid, ferric chloride, hydrochloric acid, methanol, sodium carbonate and sodium acetate.

2.2. Extraction and Determination of Flavonoids

Extraction of flavonoids was conducted using an improved ethanol flux method [20]. Two grams ground fruit sample was put in 60 mL of 60% ethanol, and the mixture was heated at 90 °C for 2 h. The extraction was repeated thrice, and the filtrates were pooled. Determination of the total flavonoid content (TFC) was carried out following a colorimetry method [21]. Absorbance (510 nm) of the flavonoid extracts was recorded with a UV–visible spectrophotometer (UV-2450, Shimadzu Corporation, Kyoto, Japan). The calibration curve was established with rutin as the standard. The TFC of samples was expressed in milligram rutin equivalents (RE) per gram of fresh fruit weight (FW, mg/g).

2.3. Flavonoid Composition Determination

Flavonoids were extracted following a method developed by Fu et al. [16] with some modifications. Fruit samples (1 g) from different stages of development were ground in liquid nitrogen and then mixed with a water–methanol–formic acid solution (80:1:19, $v/v/v$) immediately. The mixture was first sonicated for 45 min at 45 °C and then centrifuged for 10 min at 20 °C under 12,000 rpm; the supernatant was collected and mixed with 5 mL of a methanol–formic acid–water solution before filtering through a Millipore membrane.

Qualitative analysis of flavonoids was carried out using an HPLC-DAD system (Agilent 1200, Palo Alto, CA, USA), following the methods developed by Wang et al. [14]. To separate different flavonoid components, a C18 column (250 × 4.6 mm i.d.; particle size, 5 μm) was used. The binary mobile phase comprised acetic acid (2%, solvent A) and acetonitrile (solvent B). The gradient program was set as 0% to 40% solvent B for 60 min; 40% to 70% solvent B for 5 min; and 70% to 0% solvent B for 10 min. The injection volume was set as 20 μL. For simultaneous monitoring of different flavonoid compounds, the wavelengths of the detector were set at 280, 360 and 520 nm, respectively. The retention time and spectra were compared with known standards to identify individual flavonoids. The amounts of flavonoid content can be calculated using each standard calibration curve equation.

2.4. Antioxidant Determination

Flavonoid extracts from the ethanol flux method were used for antioxidant determination. The scavenging activities of DPPH (2,2-diphenyl-1-picrylhydrazyl) were measured according to Li et al. [22], and absorbance at 517 nm was then determined with a UV-2450 spectrophotometer calibrated with a trolox standard curve. The ferric-reducing antioxidant capacity (FRAP) was measured according to the method of Zheng et al. [23] by using TPTZ (2,4,6-tripyridyls-triazine) solution, and absorbance at 593 nm was then determined with the spectrophotometer, and the trolox was used as the standard. The ABTS total free radical scavenging ability was measured according to the method developed by Pereira et al. [24] by using the 2,2-azo-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and absorbance at 734 nm was then determined with the spectrophotometer, and the trolox was used as the standard. Antioxidant activity determination was repeated three times. The weight of each gram of fresh sample is expressed in mg trolox equivalent (mg TE/g).

2.5. Gene Expression Analysis

Fruit transcriptome data of *P. humilis* cultivar 'Jinou 1' were downloaded from NCBI under the accession number PRJNA417674, and nine flavonoid-synthesis-related candidate genes (*ChCHS*, *ChCHI*, *ChF3H*, *ChFLS*, *ChDFR*, *ChLDOX*, *ChUFGT, ChLAR* and *ChANR*) were screened. RNAs were prepared using the fruits of three cultivars at 5 different developmental stages (YFS, PHS, FES, CCS and FRS) using a Trizol RNA Extraction Kit (TaKaRa, China). The cDNAs were then synthesized using a high-capacity RNA-to-cDNA Kit (Applied Biosystems, Shanghai, China). Primer sequences were designed using Premier 5.0 software (PREMIER Bio-soft International, Palo Alto, CA, USA) (Table 1). Quantitative real-time PCR was performed following the method described by Han et al. [25]. The relative expression of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method [26]. Three independent biological replicates for each sample were conducted in the qRT-PCR experiment.

Table 1. Primers for quantitative real-time PCR.

P. humilis Gene ID	*P. humilis* Gene Name	Forward Primer	Reverse Primer
CL2555.Contig1	*ChCHS*	TACCAACAAGGGTGTTTCGC	GTGATCTCCGAGCACACAAC
Unigene14928	*ChCHI*	GAGGAGGAAGCCTTGGAGAA	TCCTCCTTCCCTTCAGTGTG
Unigene3562	*ChF3H*	TACAGGGAGAAGCTGTGCAA	TCACCTCTCTCCATCCCTCA
Unigene6640	*ChDFR*	TGTCGAAGAGCACCAGAAGT	GGCCAATCACAAGAGTTGGG
Unigene14860	*ChLDOX*	GGAAGGCTGGAGAAGGAAGT	TGAGCTTCAACACCAAGTGC
Unigene6233	*ChUFGT*	TGTTTGATGTGGCTGATGGC	CGTCGGTAATCAAGCAGGTG
Unigene5990	*ChLAR*	TGGCATCTCTGTGGGAGAAG	TTTCCGGTATGCGGTTCTCT
Unigene7152	*ChANR*	GAGGACCCTGAGAACGACAT	TCGTTCTCGTCTGTGACCAA
Unigene17057	*ChFLS*	GAGTTGAGGTCGTCATTGCC	TCAAGGACCCTCCCATGAAC
-	*ChActin*	GCAGCGACTGAAGACATACA	GTGGCATTAGCAAGTTCCTC

2.6. Statistical Analysis

Microsoft Excel (Version 2013) was used for statistical analyses, and correlation analyses were performed using Origin 2021 (OriginLab Corporation, Northampton, MA, USA). The differences among means were evaluated using Tukey's multiple comparison test, and differences at $p < 0.05$ were considered statistically significant. The figures were generated using GraphPad prism 8.

3. Results

3.1. Changes in Total Flavonoid Content during Fruit Development

As shown in Table 1, at the initial sampling stage (YFS), cultivar 'Jinou 1' had the highest TFC (47.21 ± 1.01 mg/g RE·FW), followed by cultivars 'Nongda 3' (22.33 ± 0.88 mg/g RE·FW) and 'Nongda 5' (12.33 ± 0.92 mg/g RE FW). The TFCs of the three cultivars all declined rapidly until fruit ripening, and significant variations ($p < 0.05$) were exhibited between fruit developmental stages (Table 2). The TFCs of ripe fruits of the three cultivars varied from 7.23 to 11.94 mg/g RE·FW, and 'Jinou 1' (a red-fruited cultivar) had the highest fruit TFC, while 'Nongda 5' (a yellow-fruited cultivar) had the lowest TFC.

Table 2. Total flavonoid changes in *Prunus humilis* fruits during fruit growth.

Developmental Stages	Nongda 3 (mg/g RE·FW)	Jinou 1 (mg/g RE·FW)	Nongda 5 (mg/g RE·FW)
YFS	22.33 ± 0.88 ^{a}	47.21 ± 1.01 ^{a}	12.33 ± 0.92 ^{a}
PHS	16.98 ± 0.99 ^{b}	24.33 ± 1.56 ^{b}	9.97 ± 1.05 ^{b}
FES	12.56 ± 0.67 ^{c}	20.65 ± 0.93 ^{c}	9.56 ± 0.98 ^{b}
CCS	11.43 ± 0.33 ^{cd}	13.23 ± 1.23 ^{d}	9.21 ± 0.65 ^{b}
FRS	10.00 ± 0.42 ^{e}	11.94 ± 0.26 ^{de}	7.23 ± 0.32 ^{c}

YFS, PHS, FES, CCS and FRS represent young-fruit stage, pit-hardening stage, fruit-enlargement stage, color-changing stage and fruit-ripening stage, respectively. The values are shown as the means ± S.D. (n = 3). The different lowercase letters within the same columns represent significant differences at $p < 0.05$.

3.2. Flavonoid Composition Variation during Fruit Development

Thirteen flavonoid compounds were detected in the fruit extracts of the three *P. humilis* cultivars using HPLC-DAD (Figure 2). Expressed in fresh weight, most flavonoid compounds were similar in their accumulation patterns. In all three cultivars, fruit development was associated with decreasing concentrations of catechin (C), proanthocyanidin B1 (PA-B1), proanthocyanidin B2 (PA-B2), phloretin-2′-O-glucoside (PG), phloretin-2′,4′-O-diglucoside (PGD), quercetin-7-O-glucoside (Q7G), quercetin-7-O-acetylglucoside (Q7acG), quercetin-3-arabinoside (Q3A), myricetin (M) and quercetin (Q). Epicatechin (EC) and phloretin-2-O-xyloglucoside (PXG) concentrations remained unchanged or increased slightly during early development, decreasing afterwards until fruit harvest. Cyanidin-3-O-glucoside (C3G) accumulation varied across cultivars, and it was not present in 'Nongda 5' from YFS to FRS, whereas C3G concentration in 'Nongda 3' and 'Jinou 1' decreased slightly during early development before increasing as the fruits matured. In the mature fruits of 'Nongda 3', 'Jinou 1' and 'Nongda 5', four compounds were found to be the main flavonoids, including PA-B1, PA-B2, PG and PDG.

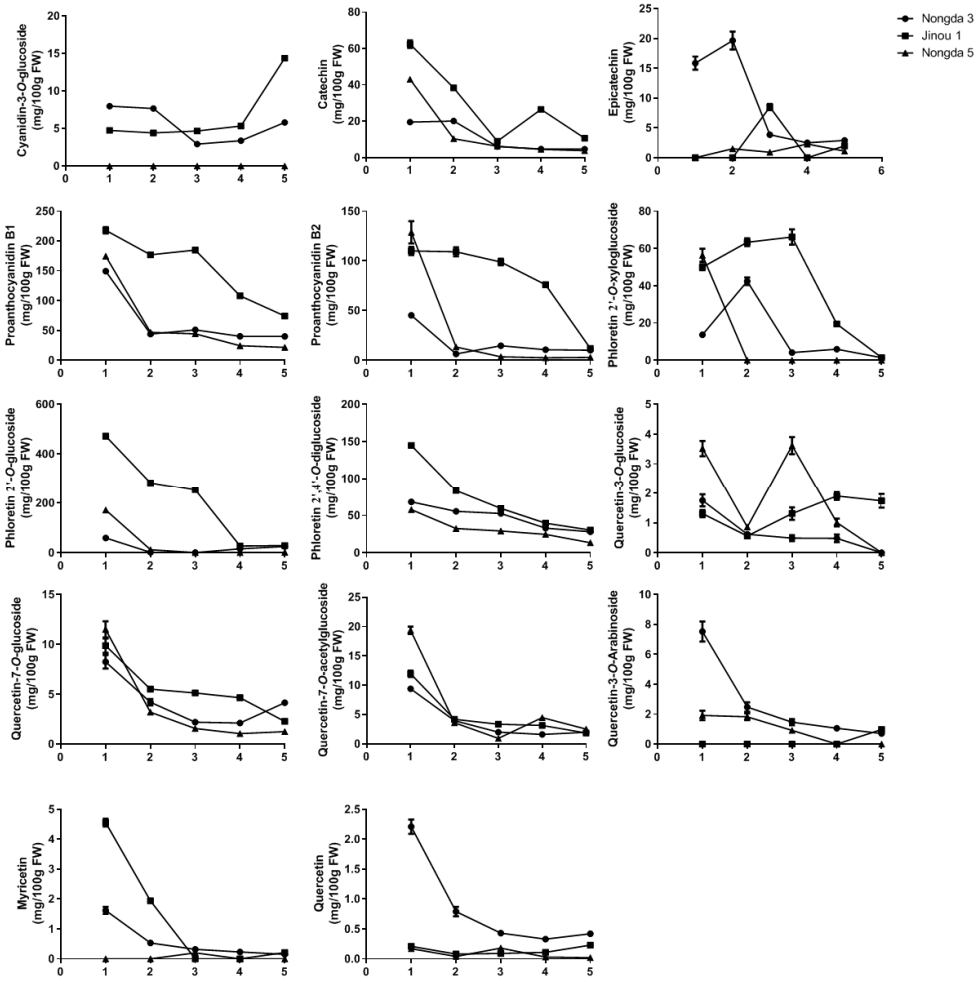

Figure 2. Accumulation of different flavonoid components in the fruits of the three *Prunus humilis* cultivars at five different developmental stages: 1, YFS (young-fruit stage); 2, PHS (pit-hardening stage); 3, FES (fruit-enlargement stage); 4, CCS (color-changing stage); and 5, FRS (fruit-ripening stage). The values are shown as the means ± S.D. (n = 3). Error bars represent standard deviations of means.

3.3. Antioxidant Activity Variation during Fruit Development

As shown in Table 3, fruits of the three *P. humilis* cultivars at the YFS showed the highest values of DPPH, FRAP and ABTS, while these values were the lowest in fruits at the stage of FRS. From YFS to PHS, antioxidant activities (DPPH, FRAP and ABTS) in fruits of the three *P. humilis* cultivars decreased slightly, while these values dropped significantly from PHS to FES ($p < 0.05$). The antioxidant activities of *P. humilis* fruits at each developing stage were all in the following order: 'Jinou 1' > 'Nongda 3' > 'Nongda 5' (Table 3). For all three cultivars, ABTS scavenging ability had the highest values, followed by FRAP and DPPH.

Table 3. Antioxidant activity changes in *Prunus humilis* fruits during fruit growth.

Cultivar	Developmental Stages	DPPH (mg TE/g)	FRAP (mg TE/g)	ABTS (mg TE/g)
Nongda 3	YFS	28.25 ± 0.39 ^{a}	53.54 ± 0.77 ^{a}	78.03 ± 1.01 ^{a}
	PHS	23.22 ± 0.41 ^{b}	45.02 ± 0.59 ^{b}	68.41 ± 0.74 ^{b}
	FES	6.13 ± 0.20 ^{c}	14.73 ± 0.34 ^{c}	31.23 ± 0.57 ^{c}
	CCS	4.10 ± 0.16 ^{d}	10.88 ± 0.44 ^{d}	9.82 ± 0.46 ^{d}
	FRS	3.33 ± 0.18 ^{e}	9.07 ± 0.50 ^{e}	8.18 ± 0.37 ^{e}
Jinou 1	YFS	36.21 ± 0.53 ^{a}	88.14 ± 1.28 ^{a}	121.02 ± 2.04 ^{a}
	PHS	35.88 ± 0.62 ^{a}	87.92 ± 1.52 ^{a}	120.41 ± 1.98 ^{a}
	FES	7.23 ± 0.14 ^{b}	18.31 ± 0.55 ^{b}	43.23 ± 0.63 ^{b}
	CCS	5.12 ± 0.11 ^{c}	16.80 ± 0.47 ^{c}	14.82 ± 0.58 ^{c}
	FRS	4.04 ± 0.22 ^{d}	11.23 ± 0.53 ^{d}	12.18 ± 0.49 ^{d}
Nongda 5	YFS	23.72 ± 0.60 ^{a}	27.21 ± 0.57 ^{a}	60.01 ± 1.04 ^{a}
	PHS	19.65 ± 0.59 ^{b}	25.76 ± 0.55 ^{b}	57.65 ± 0.89 ^{b}
	FES	5.72 ± 0.23 ^{c}	12.23 ± 0.45 ^{c}	23.31 ± 0.58 ^{c}
	CCS	3.53 ± 0.14 ^{d}	7.92 ± 0.43 ^{d}	7.44 ± 0.45 ^{d}
	FRS	2.84 ± 0.12 ^{e}	6.88 ± 0.46 ^{e}	6.57 ± 0.46 ^{e}

YFS, PHS, FES, CCS and FRS represent young-fruit stage, pit-hardening stage, fruit-enlargement stage, color-changing stage and fruit-ripening stage, respectively. The values are shown as the means ± S.D. (n = 3). The different lowercase letters within the same columns represent significant differences at $p < 0.05$.

3.4. Expression Analysis of Flavonoid Biosynthetic Genes during Fruit Development

In order to reveal the flavonoid accumulation differences of the three *P. humilis* cultivars during fruit development, the expression of flavonoid-metabolism-related genes was quantitatively verified at five different developmental stages (Figure 3). Expression of flavonoid biosynthetic genes showed different patterns in three cultivars. For yellow-fruited cultivar 'Nongda 5', expression of all nine flavonoid biosynthetic genes showed a downward trend during fruit development; for dark-red-fruited cultivar 'Jinou 1', the anthocyanin biosynthetic genes (*ChCHS*, *ChCHI*, *ChF3H*, *ChDFR*, *ChLDOX* and *ChUFGT*) were upregulated as fruit ripened, while the expressions of *ChFLS*, *ChLAR* and *ChANR* were downregulated. For yellow-red cultivar 'Nongda 3', the expression patterns of *ChCHS*, *ChCHI* and *ChF3H* decreased gradually from YFS to FES, which was similar to those of cultivar 'Nongda 5', while the expressions of these three genes increased gradually from CCS to FRS, which was similar to those of cultivar 'Jinou 1'. From CCS to FRS, fruits of 'Jinou 1' and 'Nongda 3' started to accumulate C3G (Figures 1 and 2); therefore, the expressions of *ChDFR*, *ChLDOX* and *ChUFGT* in these two cultivars were extremely upregulated (Figure 3). Interestingly, the expression level of *ChUFGT* at FRS in 'Nongda 3' was higher than that in 'Jinou 1'; however, 'Jinou 1' accumulated more C3G than 'Nongda 3' (Figures 1 and 2).

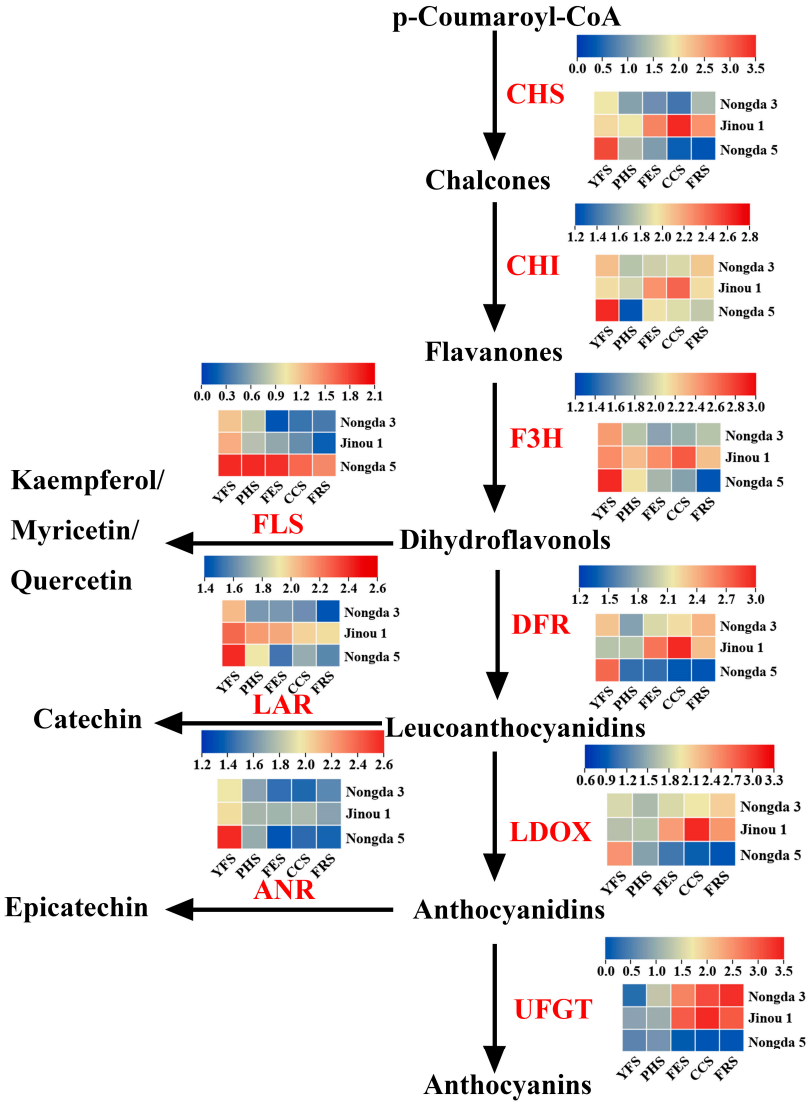

Figure 3. The flavonoid biosynthetic pathway. Enzyme names are shown in red letters, and the heatmap of coding genes was built based on their relative expression levels. Red boxes represent high expression and blue boxes represent low expression. CHS, CHI, F3H, FLS, DFR, LDOX, UFGT, LAR and ANR represent chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavanol synthase, dihydroflavonol 4-reductase, leucoanthocyanidin dioxygenase, UDP-glycose flavonoid glycosyltransferase, leucoanthocyanidin reductase and anthocyanidin reductase, respectively. YFS, PHS, FES, CCS and FRS, represent young-fruit stage, pit-hardening stage, fruit-enlargement stage, color-changing stage and fruit-ripening stage, respectively.

3.5. Correlation Analysis

By analyzing the correlations among the total flavonoid contents, flavonoid compositions, antioxidant abilities (DPPH, FRAP, ABTS) and expression of flavonoid biosynthetic genes (Figure 4), it was found that the TFCs of *P. humilis* fruits were very significantly positively correlated with antioxidant indices (DPPH, FRAP, ABTS) and the contents of catechin, PA-B1,PG, PDG and myricetin ($p < 0.01$). Except for the significant negative correlation with *ChLAR*, the TFCs had no significant correlation with the expression of most flavonoid-related genes. The expression of *ChCHS* was significantly positively correlated with *ChCHI*, *ChF3H*, *ChDFR* and *ChLDOX*. Moreover, the expression of *ChF3H* was very significantly positively correlated with catechin, PA-B1, PA-B2, Q7G and Q7acG.

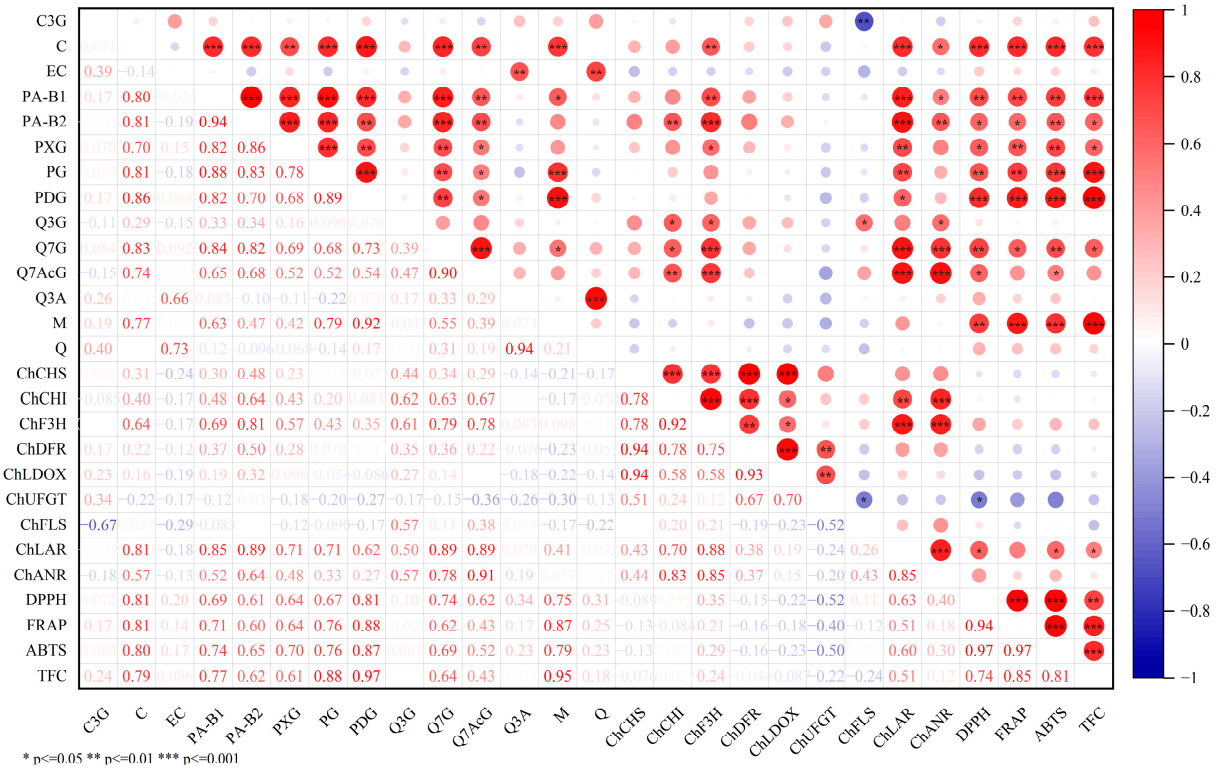

Figure 4. Correlation analysis results of the total flavonoid contents, flavonoid components, antioxidant abilities (DPPH, FRAP, ABTS) and expression of flavonoid biosynthetic genes. C3G: cyanidin-3-O-glucoside; C: catechin; EC: epicatechin; PA-B1: proanthocyanidin B1; PA-B2: proanthocyanidin B2; PXG: phloretin-2-O-xyloglucoside; PG: phloretin-2-O-glucoside; PDG: phloretin-2′,4′-O-diglucoside; Q3G: quercetin-3-O-glucoside; Q7G: quercetin-7-O-glucoside; Q7AcG: quercetin-7-O-acetylglucoside; Q3A: quercetin-3-O-arabinoside; M: myricetin; Q: quercetin; *ChCHS*: chalcone synthase; *ChCHI*: chalcone isomerase; ChF3H: flavanone 3-hydroxylase; *ChDFR*: dihydroflavonol 4-reductase; *ChLDOX*: leucoanthocyanidin dioxygenase; *ChUFGT*: UDP-glycose flavonoid glycosyltransferase; *ChFLS*: flavanol synthase; *ChLAR*: leucoanthocyanidin reductase; *ChANR*: anthocyanidin reductase; DPPH: 1,1-diphenyl-2-picryl-hydrazyl; FRAP: ferric-reducing antioxidant power; ABTS: 2,2′-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid; TFC: total flavonoid content.

4. Discussion

Flavonoids are polyphenol compounds important in plant–environment interactions, and the biosynthesis of flavonoids is upregulated in response to various biotic (pathogens, wildlife, etc.) and abiotic (UV radiation, nitrogen/phosphorus depletion, cold, salinity, drought, etc.) stresses [27,28]. In this study, the fruit TFCs of three cultivars declined rapidly from YFS to FRS. Our results agree with those from several previous studies on other fruits, which demonstrated the rapid accumulation of major flavonoids during early development before a drastic decrease in the later stages [29–31]. *P. humilis* fruits appear before the emergence of leaves and new shoots; thus, the fruits at YFS are exposed to intensive sunlight, and a high TFC in the young fruit might serve as a main factor that can protect the fruit against damage by UV light. Other reports suggested that high content of flavonoids in young fruits might also serve as a deterrent to animal feeding, since pits or seeds have not been fully developed yet and are not ready for dispersal [32]. Fruit ripening refers to changes that make fruits attractive to humans and other seed-dispersing animals, including the decline in fruit TFC [33].

The fruit TFCs exhibited significant cultivar variations ($p < 0.05$) at every fruit developmental stage examined in this study. Overall, ‘Jinou 1’ (a red-fruited cultivar) had the highest fruit TFC, while ‘Nongda 5’ (a yellow-fruited cultivar) had the lowest TFC. Generally speaking, cultivars with dark fruit color (purple, red or black) have significantly

higher TFC than light-colored cultivars (green, yellow or white) [34,35]. Although more research is necessary to determine the exact cause of such differences, phenotype is a potential selection criterion for high-TFC *P. humilis* cultivars.

The TFCs of mature *P. humilis* fruits were higher than those of sweet cherry (*Prunus avium*) cultivars and tart cherries (*Prunus cerasus*) (0.5–0.7 mg/g RE·FW) [36]. Flavonoids are the main contributors of plant antioxidant capacity and have considerable applications in the development of nutraceutical products [37]. Thus, the high flavonoid content of *P. humilis* fruit suggests that this is a promising source of natural antioxidants. Moreover, the characterization of TFC variation throughout fruit development will help to optimize fruit harvest time of *P. humilis* for maximum production of flavonoids in fruits and subsequent extraction for the food and nutraceutical industries.

Thirteen flavonoid compounds in total were detected in fruits of *P. humilis*, and twelve of them decreased during fruit development in all three cultivars; the exception was C3G. C3G was not present in cultivar 'Nongda 5' from YFS to FRS, while it accumulated in ripe fruits of cultivar 'Nongda 3' and 'Jinou 1'. C3G, as a major anthocyanin in mature fruits, is involved in plant–animal interactions, helping to attract animals to fruits by providing visual and olfactory signals [33]. C3G is also found in many other similarly colored fruits, such as blackberries, blueberries, cherries, grapes and plums [38]. Given the fact that 'Nongda 5' fruits are completely yellow, both the yellow-red 'Nongda 3' and the dark-red 'Jinou 1' fruits were expected to have relatively higher C3G concentrations.

DPPH, ABTS and FRAP methods were commonly used to determine the antioxidant activities of *P. humilis* fruit extracts. From the correlation analysis, it was found that the antioxidant abilities were very significantly correlated with the TFCs and the content of catechin, PA-B1, PG, PDG and M; therefore, these flavonoid compounds contribute the most to the antioxidant abilities and TFCs of *P. humilis* fruit. Similar results were reported in a newly published article, which stated that the proanthocyanidins (mostly PA-B1) were the main antioxidant active components of *P. humilis* fruit [39]. PG and PDG are powerful antioxidants in apple and can modulate inflammatory responses, lower blood glucose levels and also promote other aspects of health [40]. However, due to the limitation of antioxidant assay in vitro, further studies need to be conducted in vivo to verify the antioxidant abilities and other health-promoting functions of this species.

Gene expression analysis revealed that the flavonoid biosynthetic genes had different expression patterns in the three cultivars. In the red-fruited cultivar 'Jinou 1', the expression levels of *ChCHS*, *ChCHI*, *ChF3H*, *ChDFR*, *ChLDOX* and *ChUFGT* increased gradually with fruit ripening, while all flavonoid-related genes in the yellow-fruited cultivar 'Nongda 5' decreased throughout the whole process of fruit ripening. Similar results were found in sweet cherries during fruit development, and in that study, *UFGT* was suggested to be the key gene causing color differences between red and yellow cherry fruits [41]. In addition, another report revealed that the anthocyanin content in mature *P. humilis* fruit was significantly positively correlated with *ChCHS*, *ChFLS* and *ChUFGT* expression [42]. Our findings were inconsistent with the previous results that expression of *CHS* and *CHI* genes was positively correlated with flavonoid accumulation in citrus and apricot [43,44]. Although 'Nongda 5' had low expression of flavonoid biosynthetic genes and relatively lower total flavonoid contents compared to 'Jinou 1' and 'Nongda 3', its antioxidant abilities were higher than many fruit species [45], suggesting there might be some other bioactive compounds that contribute to its high antioxidant abilities.

5. Conclusions

In this study, comparative analysis revealed that the total flavonoid contents and antioxidant activities of fruits from three *P. humilis* cultivars decreased gradually during fruit development, together with the content of most flavonoid compounds. Thirteen flavonoid compounds were found in fruits of three cultivars; furthermore, cyanidin-3-O-glucoside contributed to the coloration of *P. humilis* fruits, and it accumulated only in the red-fruited cultivars 'Jinou 1' and 'Nongda 3', while it was not detected in the developing

fruits of the yellow-fruited cultivar 'Nongda 5'. The antioxidant capacities (DPPH, FRAP and ABTS) of *P. humilis* fruits showed significantly positive correlation with the TFCs and the concentrations of the main flavonoid compounds such as catechin, proanthocyanidin B1, PD and PDG. Moreover, the flavonoid biosynthetic genes had different expression patterns in the three cultivars. The expression levels of all flavonoid-related genes in cultivar 'Nongda 5' decreased gradually during fruit development, while most flavonoid-related genes increased gradually in the developing fruits of cultivar 'Jinou 1'. The results from our study will be useful for a deeper understanding of the molecular mechanisms of flavonoid accumulation and for facilitating targeted breeding of this fruit species for superior cultivars.

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