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Proanthocyanidin monomers and cyanidin 3-o-glucoside accumulation in blood-flesh peach (*Prunus persica* (l.) Batsch) fruit

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Abstract: To better understand the characteristics and mechanisms of proanthocyanidin monomers and anthocyanin synthesis in blood-flesh peach (*Prunus persica* (L.) Batsch), the accumulation of catechin, epicatechin and cyanidin 3-O-glucoside was determined, and the expression patterns of structural genes associated with biosynthesis of those compounds were investigated in the blood-flesh peach fruit of cultivar "Dahongpao" during fruit development. Our results show that catechin concentration remained low and comparatively stable throughout fruit development. The concentration of epicatechin remained low at the early stages of fruit development and rapidly accumulated during ripening. Cyanidin 3-O-glucoside was not detected in the early stages. Epicatechin started to rapidly accumulate during the ripening period, reaching a maximum at the mature stage. The expressions of the early and common genes, phenylalanine ammonia-lyase and chalcone isomerase, were less associated with proanthocyanidin monomers and cyanidin 3-O-glucoside accumulation. The expression of other flavonoid 'early' biosynthetic genes, including chalcone synthase (CHS), flavanone 3-hydroxylase, dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX), were partly associated with proanthocyanidin monomers and cyanidin 3-O-glucoside levels, with expression quantities peaking synchronously at the mature stage. Leucoanthocyanidin reductase and anthocyanidin reductase, which were the key genes for proanthocyanidin monomer synthesis, correlated during fruit development with catechin and epicatechin accumulation respectively; UDP-glucose: flavonoid 3-O-glucosyltransferase (UGFT), the key gene for anthocyanin synthesis, was correlated with cyanidin 3-O-glucoside levels. The synchronous accumulation of epicatechin and cyanidin 3-O-glucoside in blood-flesh peach could not be explained by the current theory of competitive distribution mechanism of common substrate.

Key words: blood-flesh peach (*Prunus persica* (L.) Batsch); catechin; epicatechin; cyanidin 3-O-glucoside; gene expression

INTRODUCTION

Proanthocyanidin and anthocyanin in fruit are increasingly recognized as producing health beneficial effects in humans. At the same time, proanthocyanidin possesses astringency and flavor, and is a major quality factor for fruit, while anthocyanin improves visual appeal and enhances the commercial value of fruit [1,2]. Therefore, data collection and theoretical research on the composition, accumulation and synthetic mechanism of proanthocyanidin and anthocyanin in fruit are of great significance for breeding new cultivars rich in beneficial ingredients.

The biosynthesis of proanthocyanidin and anthocyanin (Fig. 1), initiated from phenylalanine, is channeled into the flavonoid pathway by chalcone synthase (CHS). A further enzymatic reaction, catalyzed by dihydroflavonol 4-reductase (DFR), allows the formation of substrate. Leucoanthocyanidin dioxygenase/anthocyanidin synthase (LDOX/ANS) leads to the synthesis of anthocyanidin [3]. Leucoanthocyanidin reductase (LAR) provides a pathway for the synthesis of primer or extension units for proanthocyanidin, such as catechin, which is one of the proanthocyanidin monomers [4]. While UDP-glucose flavonoid-3-O-glucosyltransferase (UFGT) leads to the synthesis of anthocyanin, such as cyanidin 3-O-glucoside [5], anthocyanidin reductase (ANR) provides another pathway for the synthesis of another proanthocyanidin monomer, epicatechin [4]. The common genes

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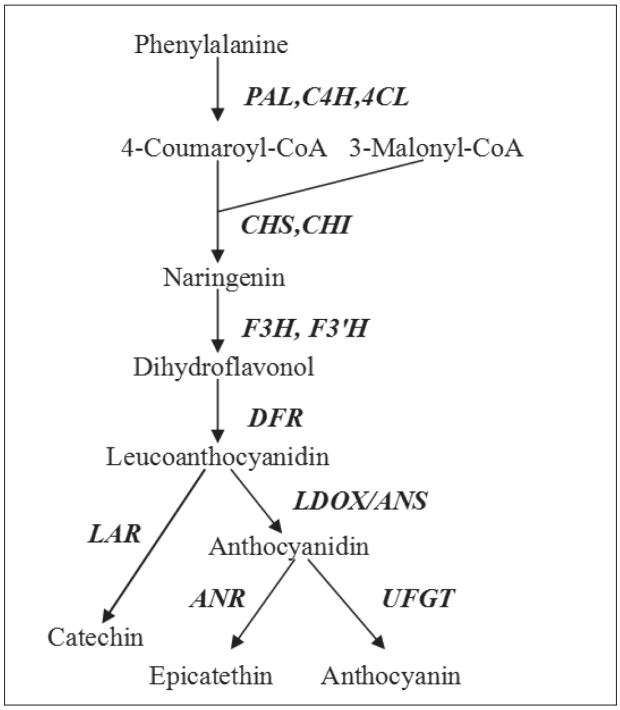

Fig. 1. Proanthocyanidin and anthocyanin biosynthetic pathway in plants [1, 6]. Structural genes for each step are indicated as follows: *PAL*, phenylalanine ammonia-lyase, *C4H*, cinnamate 4-hydroxylase, *4CL*, 4-coumarate-CoA ligase, *CHS*, chalcone synthase, *CHI*, chalcone isomerase, *F3H*, flavanone 3-hydroxylase, *F3'H*, flavanone 3'-hydroxylase, *DFR*, dihydroflavonol 4-reductase, *LDOX*, leucoanthocyanidin dioxygenase, *ANS*, anthocyanins synthetase, *UFGT*, UDP-glucose: flavonoid 3-O-glucosyltransferase, *LAR*, leucoanthocyanidin reductase, and *ANR*, anthocyanidin reductase.

regulating the synthesis of proanthocyanidin and anthocyanin are phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate-CoA ligase (*4CL*), CHS, chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), F3'H, DFR, anthocyanins synthetase (*ANS*) and *LDOX*. *UFGT* is a key gene for the synthesis of cyanidin 3-O-glucoside, while *LAR* and *ANR* are the key genes for proanthocyanidin monomers [1,6].

Blood-flesh peach (*Prunus persica* (L.) Batsch) belongs to the subfamily Prunoideae in the Rosaceae family. It is an important type of peach germplasm with red fruit-flesh color, which is rich in proanthocyanidins and anthocyanins [7-10]. Chlorogenic acid, neochlorogenic acid, catechin, epicatechin, rutin, quercetin, gallic acid, ferulic acid, phlorizin and phloretin have been detected in blood-flesh peach, but the proanthocyanidin monomers, including catechin and epicatechin are the main components [10,11], while the main anthocyanin is cyanidin 3-O-glucoside [12,13]. Cyanidin 3-O-glucoside accumulation and its mechanism have been widely studied [13-17]. However, little is known about the metabolic mechanism of proanthocyanidin monomer metabolism in blood-flesh peach because all existing research has been conducted on white-flesh peach [18-20].

In the current paper, the blood-flesh peach cultivar "Dahongpao" was researched to better understand the characteristics and mechanisms of proanthocyanidin monomers (catechin and epicatechin) and cyanidin 3-O-glucoside accumulation. We investigated in detail the accumulation of catechin, epicatechin and cyanidin 3-O-glucoside in blood-flesh peach during fruit development, and identified the expression of structural genes encoding the proanthocyanidin monomers and cyanidin 3-O-glucoside biosynthesis enzymes, including *PAL*, *CHS*, *CHI*, *F3H*, *DFR*, *LDOX*, *UFGT*, *LAR* and *ANR*. We discuss the relationship between the accumulation of these proanthocyanidin monomers and cyanidin 3-O-glucoside. The results of our research could be very useful for further study of the proanthocyanidin and anthocyanin synthesis mechanism in peach.

MATERIALS AND METHODS

Plant materials and experimental treatments

Experiments were conducted using seven-year old trees of blood-flesh peach (cultivar "Dahongpao") maintained at the National Peach Germplasm Repository (Nanjing, China) in 2014. Fruit samples were collected from six trees every 7 days, beginning at 30 DAFB (days after full bloom). And each stage consisted 18 fruits with three replicates. Fruit was mechanically peeled and cored and the flesh cut into small sections. Fruit samples at each stage were mixed and immediately frozen in liquid nitrogen and stored at -75°C until use. According to the method of Lombardo et al [21], the sampling points were confirmed through the growth curve of a double-S form fitted to fruit weight. Specifically, the sampling points were 30 (S1), 58 (S2), 79 (S3), 93 (S4) and 100 (H) DAFB.

Extraction and measurement of catechin, epicatechin and cyanidin 3-O-glucoside

Catechin and epicatechin were extracted and measured according to Yan et al [11]. Frozen fruit (1 g) was homogenized in 2 mL of methanol containing 0.1% H_3PO_4, and the extracts were centrifuged at 10000*x*g for 5 min at 4°C, and filtered through a 0.22-μm filter for analysis in the Agilent 1100 series HPLC system (Agilent, USA). Samples (5 μL of extract) were analyzed using an Agilent ZORBAX SB-C18 column (4.6×250 mm, 5 μm) coupled with a UV detector at 280 nm, with ethanol (0.1% H_3PO_4) and water (0.1% H_3PO_4) as solvents: the flow rate was 1.0 mL·min^{-1} and temperature was 30°C.

Cyanidin 3-O-glucoside was extracted and measured according Yan et al [22]. Frozen fruit (1 g) was homogenized in 2 mL of extracting solution [methanol:water:phosphate:trifluoroacetic acid = 70:27:2:1 (V/V)], and the extracts were centrifuged at 10000 *x*g for 5 min at 4°C and filtered through a 0.22-μm filter for analysis in the Agilent 1100 series HPLC system. Samples (5 μL of extract) were analyzed using an Agilent ZORBAX SB-C18 column (4.6×250 mm, 5 μm) coupled with a UV detector at 525 nm, with methanol:phosphate:trifluoroacetic acid = 97:2:1 (V/V) and water:phosphate:trifluoroacetic acid = 97:2:1 (V/V) as solvents: the flow rate was 1.0 mL·min^{-1} and temperature was 25°C.

Catechin, epicatechin and cyanidin 3-O-glucoside, and HPLC-grade methanol, acetic acid, phosphoric acid and trifluoroacetic acid were purchased from Sigma-Aldrich (Shanghai, China). Aqueous solutions were prepared using ultra-pure water purified by Milli-Q System (S.A.S. 67120, Millipore, Molsheim, France). Compounds were quantified by comparing the peak areas and presented as mg of catechin or epicatechin or cyanidin 3-O-glucoside per g of fresh tissue (mg·kg^{-1} FW).

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

RNA was extracted from frozen flesh obtained from "Dahongpao" using a modified CTAB method [14]. After the removal of DNA by DNase I, the concentration of total RNA was measured and the first cDNA strand was synthesized from 1 μg of total RNA using Supersmo III M-MuLV Reverse Transcriptase (Bioteke Corporation, Beijing, China) primed with oligo (dT) 18. The cDNA was diluted 50× and 2 μL of the diluted cDNA was used as the template for qRT-PCR analysis. We designed qRT-PCR primers with Oligo 7.37 and Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the obtained sequences of cDNA fragments. RT-PCR analysis was performed using the primers presented in Table 1 [14,18,23]. qRT-PCR was performed on an ABI 7500 System (Applied Biosystems, Foster, CA, USA) using the SYBR Premix Ex Taq™ (Takara, Japan). The PCR reaction consisted of 10 μL of SYBR Green PCR Master Mix, 0.8 μL of forward and reverse primer (10 μM), 0.4 μL of ROX Reference dye (50x; all from Takara), 6.0 μL of dH_2O and 2.0 μL of 1:50-diluted template cDNA in a total volume of 20 μL. The two-step RT-PCR program was initiated with a preliminary step at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s. The assay included a no-template control for each primer pair. All qRT-PCR reactions were normalized using the Ct value corresponding to *actin*. Three measurements for each biological replicate sample were performed.

Table 1. Primers for quantitative real-time PCR

Gene	Accession No.	Forward primers (5'–3')	Reverse primers (5'–3')
PAL	HM543574	AAGCTGCTGAAAAGGTGCAT	TCATTTTGGTTGCTGCTCTG
CHS	AB094986	CAGAGATACCCAAAGGTTGGAAGGC	AACCATCCTTCCCGACAGCGAT
CHI	DY634915	TGAAGACCTCAAGGAACTTCTCAATGG	ACACAGGTGACAACGATACTGCCACT
F3H	AB097151	TCCGAGGGCAGAGCGAAGAAC	TTGTGGAGGCTTGTGAGGATTGG
DFR	AB095030	GGTCGTCCAGGTGAACATACTGCC	ATTTCTCATGCCATCCATGCCAC
LDOX	EU292219	AAGTGGGTCACTGCCAAGTGTGTTC	GTGGCTCACAGAAAACTGCCCAT
UFGT	DN676790	CCGCTGCCTCTCCCAACACTC	CCATCAGCCACATCAAACACCTTTAT
ANR	AM288300	ACTTCAAGGCTAAGGGGCTGCTG	CCAAGCCAGATAAACGCCAATCAC
LAR	AJ872926	CATCCACGGGGAAATTCACCTG	ACCCTTCCCAGAGTTACCATCACTGA
Actin	TC1223	GTTATTCTTCATCGGCGTCTTCG	CTTCACCATTCCAGTTCCATTGTC

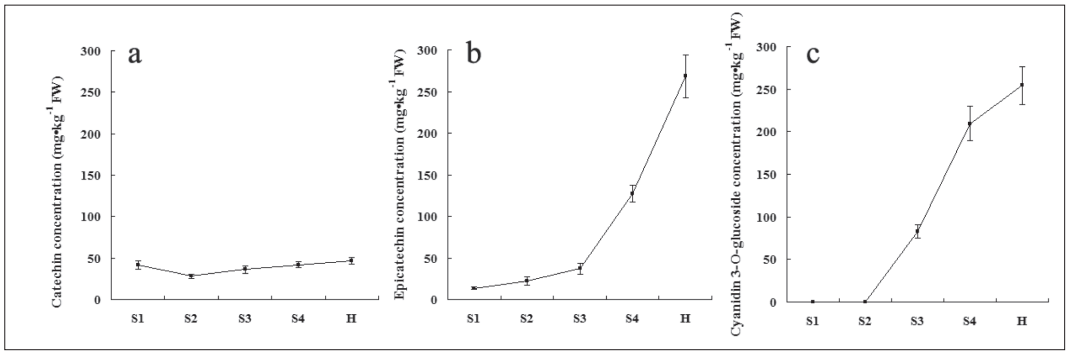

Fig. 2. Proanthocyanidin and anthocyanin concentration changes in flesh of blood-flesh peach. Values are means±standard error. S1, S2, S3, S4, H were the sampling points at 30, 58, 79, 93 and 100 days after full bloom, respectively. **a** – Catechin concentration changes; **b** – epicatechin concentration changes; **c** – cyanidin 3-O-glucoside concentration changes.

Statistical analysis

Figures were drawn with Microsoft Excel 2010 (Microsoft corp., Northampton, MA, USA), and least significant differences were calculated for mean separations using a *t*-test of the Data Processing System (DPS, version 14.10, Zhejiang University, Hangzhou, China).

RESULTS

Catechin, epicatechin and cyanidin 3-O-glucoside accumulation during fruit development

In the blood-flesh peach "Dahongpao", the concentration ranges of catechin, epicatechin and cyanidin 3-O-glucoside were 28.00-44.42, 13.45-268.20 and 0-254.42 mg·kg^{-1} FW, respectively (Fig. 2). This result pointed to significant differences in proanthocyanidin and anthocyanin concentrations in blood-flesh peach. Moreover, the dynamic change trends of catechin and epicatechin concentrations significantly differed. At the early and middle stages of fruit development, the concentrations of catechin were slightly higher than those of epicatechin, while in the fruit maturation period the concentration of epicatechin was significantly higher than that of catechin ($p<0.01$). The catechin concentration remained low throughout fruit development, and even declined at stage S2 ($p<0.01$); it increased during the middle stages ($p<0.01$) and remained stable in the ripening period, with a slight but non-significant increase. The concentration of epicatechin remained low at the early stages of fruit development, exhibiting rapid accumulation from stage S3 to a peak at stage H, with a concentration of 268.20 mg·kg^{-1} FW ($p<0.01$). The dynamic change trend of cyanidin 3-O-glucoside was only slightly different from that of epicatechin. Cyanidin 3-O-glucoside was not detected at the early stages of fruit development, however, it started to accumulate at stage S3 and rapidly accumulated from stage S3 to a peak at H, with a concentration of 254.42 mg·kg^{-1} FW ($p<0.01$).

Expression of structural genes during fruit development

There were significant differences in the levels of expression of structural genes during fruit development in "Dahongpao" (Fig. 3). The upstream region of genes *PAL*, *CHS*, *CHI*, *F3H*, *DFR* and *LDOX* will be discussed first. The expression of *PAL* and *CHI* was low and at about equivalent levels at different stages. The level of expression of *CHS* at the transcriptional level decreased gradually from stage S1 to a minimum that was observed at S3 ($p<0.01$); it then increased sharply at the ripening stages and attained a maximum at stage H ($p<0.01$). The level of *F3H* expression was similar to that of *CHS*, with a slight decrease at the middle stage and a significant rise at stage H ($p<0.01$). The level of *DFR* expression increased slightly and remained stable at the middle stage. As for *CHS* and *F3H*, there was a significant rise at stage H ($p<0.01$). The levels of both *LDOX* and *UFGT* expression increased gradually from stage S1 and attained a maximum at stage H ($p<0.01$). The level of *LAR* expres-

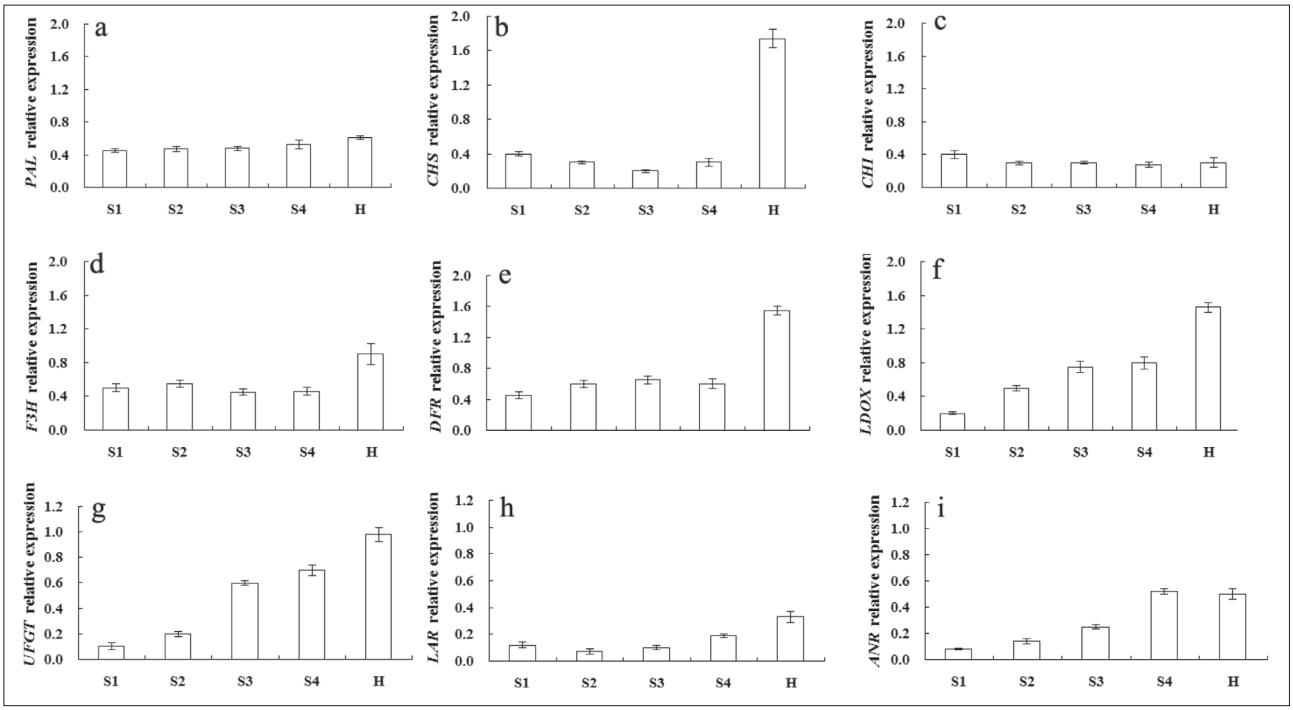

Fig. 3. Relative expression of genes during blood-flesh peach fruit development. Values are means±standard error. S1, S2, S3, S4, H were the sampling points at 30, 58, 79, 93 and 100 days after full bloom, respectively. **a** – *PAL* relative expression; **b** – *CHS* relative expression; **c** – *CHI* relative expression; **d** – *F3H* relative expression; **e** – *DFR* relative expression; **f** – *LDOX* relative expression; **g** – *UFGT* relative expression; **h** – *LAR* relative expression; **i** – *ANR* relative expression.

sion did not significantly differ from stage S1 to S3; it slightly decreased at stages S2 and S3 and markedly increased from stage S4 to H ($p<0.01$). The expression of *ANR* significantly increased with the stages of fruit development, and reached a stable maximum in the ripening period ($p<0.01$).

DISCUSSION

The structure, function and synthetic mechanism of plant secondary metabolites have attracted attention in recent years. Anthocyanin function and biosynthesis and gene organization, expression and regulation have been investigated, however, there has been little research into proanthocyanidin [6,24]. We determined the accumulation of proanthocyanidin monomers (catechin and epicatechin) and cyanidin 3-O-glucoside in blood-flesh peach "Dahongpao", and found that the concentration of catechin remained low and almost stable throughout fruit development. The concentration of epicatechin remained low at the early stages of fruit development and it rapidly accumulated to a maximum at the matured stage, while cyanidin 3-O-glucoside was not detected in the early stages of fruit development. As for epicatechin, it accumulated rapidly during the ripening period to a maximum at the mature stage. The developmental profiles of catechin and epicatechin accumulation were different from those reported in previous research, in which their accumulation decreased with fruit maturation [18,19]. Cyanidin 3-O-glucoside accumulation increased with fruit maturation, and the result was consistent with previous research [14].

Until now, there has been no clear definition of the major genes regulating the synthesis of proanthocyanidins and anthocyanins in peach. Tsuda et al [17] found that *CFS* and *DFR* are the key genes regulating the synthesis of anthocyanins in blood-flesh peach. Zhao et al [13] suggested that *PAL* was the key enzyme for anthocyanin synthesis. Daniela et al [18] established that only *UFGT* was weakly correlated with anthocyanin level, while the expression of structural genes *CHS*, *CHI*, *FSH*, *DFR*, *LDOX*, *UFGT*, *ANR* and *LAR* correlated with proanthocyanidin accumulation. Jiao et al [14] found that cyanidin 3-O-glucoside accumulation in the fruit of blood-flesh peach "Banjintao"

was closely related to the coordinated expression of *UFGT* and *ANS*. In white-flesh peach, Daniela et al [18] proposed that the expression of the genes encoding enzymes of the flavonoid pathway, especially *LAR* and *ANR*, correlated with proanthocyanidin concentration. Zhou et al [19] suggested that *MYB7* activated transcription of *LAR*, but not *ANR*; however, in the same research, the expression of *LAR* and *ANR* could not adequately explain the dynamic changes in catechin and epicatechin concentrations during fruit development. In our research on blood-flesh peach "Dahongpao", the expression of the upstream region of *PAL* and *CHI* were less associated with proanthocyanidin monomers and cyanidin 3-O-glucoside accumulations, while the expression of other genes, including *CHS*, *F3H*, *DFR* and *LDOX*, were partly associated with proanthocyanidin monomers. Cyanidin 3-O-glucoside levels and the expression levels peaked synchronously in ripe fruit. Moreover, the expression of *UFGT*, the key gene for anthocyanin synthesis, was highly correlated with cyanidin 3-O-glucoside levels, while the expression of *LAR* and *ANR*, the key genes for proanthocyanidin synthesis, correlated with catechin and epicatechin accumulation during fruit development, respectively. We conclude that *LAR*, *ANR* and *UFGT* are key genes regulating the synthesis of proanthocyanidin monomers and cyanidin 3-O-glucoside because the levels of their expression correlated with the levels of these compounds. However, the expression of the upstream region of common genes such as *CHS*, *DFR* and *LDOX*, significantly increased with fruit development and maturation. This could give rise to the accumulation of a common substrate used in proanthocyanidin monomer and cyanidin 3-O-glucoside synthesis. Thus, *CHS*, *DFR* and *LDOX* might be major genes regulating the synthesis of epicatechin and cyanidin 3-O-glucoside in blood-flesh peach.

Interestingly, we found synchronous accumulation of epicatechin and cyanidin 3-O-glucoside during the fruit-ripening period, which was very different from other fruits and could not be explained by the current mechanism of competitive distribution of a common substrate [25]. There were obviously negative correlations between the accumulation of epicatechin and cyanidin 3-O-glucoside during fruit development in previous research conducted on colored fruits: grape [26], blueberry [27], bilberry [28], strawberry [29] and blackberry [30]. Cyanidin 3-O-glucoside, which remained at a low concentration during the early developing stage, increased dramatically as the fruit matured. In contrast, epicatechin exhibited a continuously decreasing pattern. Accordingly, the transcript levels of genes specifically controlling either of these two compounds, *UFGT* anthocyanin and *LAR* and *ANR* proanthocyanidin, generally coordinated with the changing patterns of products. These findings imply that the mechanism of synchronous accumulation of epicatechin and cyanidin 3-O-glucoside in blood-flesh peach should be researched further.

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Conflict of interest disclosure: The authors have declared that no conflict of interests exists.

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