



Article

Metabolic Profiles of Pomegranate Juices during Fruit Development and the Redirection of Flavonoid Metabolism

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Abstract: The pomegranate (*Punica granatum* L.) fruit is favorable for its nutrient-rich benefits to human health. However, the global metabolic profiles of pomegranate juice and the metabolic mechanisms of its essential metabolites are poorly understood. In this study, we conducted a widely targeted metabolome, integrated with the transcriptome of juices (edible parts) of pomegranate fruits at 50, 95, and 140 days after flowering (DAF) to comprehensively investigate the metabolic profiles and potential metabolism of essential metabolites. Five hundred and nine metabolites, including 11 sugar and sugar alcohols, 17 common organic acids, 20 essential amino acids, and a variety of flavonoids, were detected in pomegranate juices. Among them, metabolites in the flavonoid biosynthesis pathway greatly changed during fruit development. Notably, the redirection of metabolite flux from catechin and its derivative synthesis to anthocyanin synthesis occurred at the later developmental stages. The increased expression of *Pgr021399.1* encoding dihydroflavonol 4-reductase (DFR), *Pgr017842.1* encoding anthocyanidin synthesis (ANS), *Pgr015322.1* encoding anthocyanidin 3-O-glucosyltransferase (BZ1), *Pgr000447.1* encoding UTG75C1, and the decreased expression of *Pgr024128.1* encoding leucoanthocyanidin reductase (LAR) may trigger redirection. The results of this study provide a global view of the metabolic profiles of pomegranate juices and valuable information on the molecular mechanisms underlying the redirection of flavonoid metabolism. It also sheds light on the genetic regulation of flavonoid metabolism in pomegranate juices.

Keywords: metabolic profiles; metabolite flux redirection; flavonoid biosynthesis; pomegranate



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1. Introduction

Pomegranate (*Punica granatum* L.) is a perennial fruit tree cultivated worldwide for its special flavor and excellent nutrition, which is beneficial to human health [1]. Pomegranate fruit is an excellent source of bioactive polyphenols, including flavonoids (flavonols, flavones, flavanones, flavan-3-ols, isoflavones, and anthocyanins), hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic acid, and ellagic acid esters of glucose), sugars, and organic acids [2]. Pomegranate is marketed as a fresh fruit, juice, syrup (grenadine), wine, and other products [3]. Many studies have indicated that bioactive compounds in pomegranate help to fight cancer [4], reduce hypertension [5], boost heart health [6], relieve osteoarthritis [7], fight inflammation [8], and provide lots of antioxidants [9]. Pomegranate is used in traditional medicine in several regions [10]. To date, 137 clinical trials of pomegranate juice on human health have been registered (<https://clinicaltrials.gov/ct2/results?term=pomegranate>,

accessed on 29 June 2023). Although a variety of metabolites has been detected in pomegranate juice (<https://nutritiondata.self.com>, accessed on 29 June 2023), a global metabolic profile of pomegranate juice is still lacking. The developing phenolic compounds endowed the alternative color and flavor of pomegranate fruit during development, while its metabolic mechanism is still unknown.

Flavonoids are a group of phenolic compounds, which play an important role in human health due to their antimicrobial, antioxidant, anti-inflammatory, and anticancer properties [11]. Flavonoids are the main bioactive compounds in pomegranate, which are derived from the phenylpropanoid biosynthesis pathway. They are synthesized from p-coumaroyl-CoA by chalcone synthase (CHS) and chalcone isomerase (CHI), followed by reduction and hydroxylation reactions. Naringenin is an important intermediate of flavonoids biosynthesis pathway, which is further hydroxylated by flavanone 3-hydroxylase (F3H) to generate dihydrokaempferol, and then catalyzed by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) to yield dihydroquercetin and dihydromyricetin, respectively. Dihydrokaempferol, dihydroquercetin, and dihydromyricetin are collectively referred to as dihydroflavonols, which can be further converted to different types of flavonols by flavonol synthase (FLS). Alternatively, dihydroflavonols can also be utilized by dihydroflavonol 4-reductase (DFR) to produce leucoanthocyanidins. These colorless leucoanthocyanidins are then transformed into stable-colored anthocyanins catalyzed by anthocyanidin synthesis (ANS). Anthocyanins can be transformed into anthocyanidins catalyzed by UDP-glucose: anthocyanin 3-O-glucosyltransferase, UDP-glucose: anthocyanin 5-O-glucosyltransferase, and anthocyanidin 3-O-glucoside 5-O-glucosyltransferase. On the other hand, leucoanthocyanidins and anthocyanidins can also be converted to proanthocyanidins, the polymers of catechin, epicatechin, gallocatechin, epigallocatechin, and so on, catalyzed by leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) [12]. Metabolite constitution and accumulation in plants mainly depend on genetics. Understanding the molecular mechanism of metabolite flux redirection (MFR) is fundamental for understanding the molecular regulation of some essential metabolites.

P. granatum 'Dabenzi' and *P. granatum* 'Tunisia' are the most popular cultivars in China and are mostly consumed as fresh fruit. Both varieties display red fruits at maturity, have distinct taste and flavor profiles. We completed the metabolic profiling of juices (edible parts) of the cultivar at different growth stages (i.e., 50, 95, and 140 days after flowering) to investigate the characteristics of metabolite accumulation at different developmental stages, and obtained the essential metabolites accumulated in juice at the later developmental stage. Then, we carried out studies on the genetic mechanism of essential metabolite biosynthesis. The results not only provide information on the metabolic profiles of pomegranate juices but also shed light on the genetic regulation of flavonoid synthesis.

2. Materials and Methods

2.1. Plant Materials

Three trees of the same age and growth status from each of the two varieties, *P. granatum* 'Dabenzi' and *P. granatum* 'Tunisia', planted in a commercial orchard in Hefei (31°51'9.05" N, 117°06'34.33" E), Anhui Province of China, and grown under the same cultivation conditions, including fertilization and irrigation, were selected. Nine fruits were harvested from each tree at 35, 50, 75, 95, 105, 125, and 140 DAF and pooled as one replicate for the determination of the chemical parameters of the juices. Juices of fruits at 50, 95, and 140 DAF were selected as representative examples of early, middle, and late developmental stages to carry out metabolism and transcript analysis. The juice was acquired by squeezing the outer seed coat, as described by Qin (2020) [13], frozen immediately in liquid nitrogen, and stored at −80 °C for further use.

2.2. Determination of Chemical Parameters of Pomegranate Juices

Total soluble solids (TSS) were determined using the sulphuric acid-anthrone colorimetric method [14]. Titratable acid (TA) was determined by titration against 0.1 mol/L

NaOH using phenolphthalein as an endpoint indicator and expressed as the number of millimoles of hydrogen ions per 100 g juice [15]. The total phenolic content was determined using the Folin–Ciocalteu colorimetric method [16]. Total tannin content was measured by the Folin Denis method [17], which is based on the measurement of blue color formed by the reduction in phosphotungstomolybdic acid by tannin-like compounds in an alkaline solution. Total anthocyanins were extracted as described by Lee and Wicker [18]. A total of 0.3 mL juice was extracted with 1.5 mL 1% methanol hydrochloric acid for 4 h in the dark at 4 °C and then centrifuged at 12,000 r/min for 10 min at 4 °C. The absorbance of each sample was measured at 530, 620, and 650 nm using a spectrophotometer. The total anthocyanin content was determined using the following formula: $OD = (A_{530} - A_{620}) - 0.1(A_{650} - A_{620})$. The spectrophotometer used in the study was a UV–VIS spectrophotometer (UV-6100, Metash Instruments Co., Ltd., Shanghai, China). Titrator was Digital Titrette–A (Brand Trading Co., Ltd., Wertheim, Germany). Reagents were guaranteed pure or chemically pure, which were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.3. Analysis of Metabolome and Transcriptome

Widely targeted metabolites were identified using liquid chromatography (LC) and electrospray ionization (ESI) mass spectrometry (MS) system equipped with a C18 column (LC, Shim-pack UFLC Shimadzu CBM30A system; ESI, MS, Applied Biosystems 6500 QTRAP®, AB Sciex, Foster City, CA, USA, C18 column, Waters ACQUITY UPLC HSS T3). Applied Biosystems 6500 QTRAP® (AB Sciex, Foster City, CA, USA) was equipped with linear ion trap (LIT) and triple quadrupole (QQQ) scans. The analytical conditions were as described previously [13]. A qualitative analysis of MS data was carried out by comparison with standards or the database MWDB (MetWare Biological Science and Technology Co., Ltd., Wuhan, China) or publicly available metabolite databases if the standards were unavailable. A quantitative analysis was performed based on the chromatographic peak area. Differential metabolites were defined as metabolites when the fold change (FC) value was ≥ 2.0 or ≤ 0.5 . An enrichment analysis of differential metabolites was performed using the Kyoto Encyclopedia of Genes and Genomes Orthology (<http://www.genome.jp/kegg/ko.html>, accessed on 29 June 2023) with an E value of $\leq 1 \times 10^{-10}$. Venn diagrams, volcano plots, and heat maps of differential metabolites were generated using MetaboAnalyst (Version 4.0) (<https://www.metaboanalyst.ca>, accessed on 29 June 2023). For normalization, the relative contents of metabolites were log₂-transformed.

The transcriptome data were collected from the NCBI Sequence Read Archive under accession number RJNA548841. Differentially expression genes (DEGs) were selected with fragments per kilobase of transcript per million mapped reads (FPKM) > 1 , fold change (FC) > 2.0 , and adjusted *p*-value < 0.001 . KEGG enrichment analysis of DEGs was performed with an E value of $\leq 1 \times 10^{-10}$. The values of FPKM were scaled to Z-scores to draw the heatmap. The data of gene expression and metabolite contents were visualized by drawing a heatmap using TBtools software v0.665 (Guangzhou, China).

2.4. Statistical Analysis

The statistical significance was calculated by Student's test using the SPSS 13.0 software (SPSS, Chicago, IL, USA).

3. Results

3.1. Chemical Parameters of Pomegranate Juices during Fruit Development

TSS, TA, phenolics, and tannins are quality attributes that reflect fruit taste and nutrition. In pomegranate juices, TSS contents gradually increased with fruit development, whereas TA contents decreased at the early developmental stages, and then maintained low levels (Table 1). It was earlier for anthocyanins biosynthesis of 'Tunisia' compared to 'Dabenzi', and the accumulation of ripe fruits was earlier for 'Tunisia' compared to 'Dabenzi'. Total phenolic and tannin contents in pomegranate juices were high at the early developmental stage and then decreased with fruit development. The contents of TSS, TA, total anthocyanins, phenolics, and tannins changed gradually after 95 DAF.

Table 1. Chemical properties of pomegranate juices during fruit development.

Metabolite Name	<i>P. granatum</i> 'Dabenzi'						<i>P. granatum</i> 'Tunisia'							
	35 d	50 d	70 d	95 d	105 d	120 d	140 d	35 d	50 d	70 d	95 d	105 d	120 d	140 d
Soluble sugar (µg/mL)	8.21 ± 0.34	10.68 ± 0.05	12.28 ± 0.02	13.45 ± 0.51	16.26 ± 0.89	18.50 ± 0.19	20.19 ± 0.68	7.27 ± 0.96	9.52 ± 0.51	13.26 ± 1.24	15.62 ± 0.53	16.86 ± 0.69	18.62 ± 0.22	19.37 ± 0.66
Total titratable acidity (%)	0.65 ± 0.02	0.61 ± 0.03	0.62 ± 0.01	0.47 ± 0.35	0.47 ± 0.01	0.47 ± 0.01	0.40 ± 0.02	0.48 ± 0.02	0.38 ± 0.01	0.29 ± 0.02	0.28 ± 0.01	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.03
Total anthocyanin (mg/L)	5.33 ± 0.17	10.10 ± 0.01	16.70 ± 1.21	39.07 ± 5.40	104.03 ± 2.90	138.87 ± 10.82	173.60 ± 7.46	5.33 ± 0.16	48.40 ± 7.00	106.40 ± 7.41	168.27 ± 1.53	167.17 ± 2.85	182.50 ± 5.38	195.17 ± 1.25
Total phenolics (mg/mL)	5.81 ± 0.35	5.13 ± 0.32	4.21 ± 0.07	2.55 ± 0.03	2.37 ± 0.04	2.18 ± 0.03	2.02 ± 0.11	3.66 ± 0.11	2.60 ± 0.04	2.16 ± 0.04	1.82 ± 0.04	1.47 ± 0.04	1.34 ± 0.06	1.51 ± 0.04
Tannins (mg/mL)	5.20 ± 0.08	5.14 ± 0.09	5.33 ± 0.03	3.03 ± 0.07	2.84 ± 0.02	2.58 ± 0.06	2.41 ± 0.02	5.09 ± 0.08	3.63 ± 0.09	3.35 ± 0.03	2.56 ± 0.07	2.41 ± 0.02	1.57 ± 0.06	1.23 ± 0.02

3.2. Metabolic Profiling of Pomegranate Juices during Fruit Development

In total, 509 metabolites constituted of primary and secondary metabolites were detected in pomegranate juices (Table S1). There were seven sugars, four sugar alcohols, 16 common organic acids, 20 essential amino acids, six alkaloids, 26 flavonols, 15 flavanones, 19 flavones, 11 catechins and their derivatives, 10 anthocyanins, 4 pro-anthocyanidins, etc. The metabolites had different accumulation patterns in different cultivars at different developmental stages. D-(+)-glucose and D-(+)-sucrose were the main sugars in the juice of ripe fruits, followed by DL-arabinose and D-fucose. It is known that in plants, sugars such as D-(+)-glucose and D-fucose can be converted into sugar alcohols, such as D-sorbitol and D-mannitol. In this study, we detected lots of D-mannitol, D-sorbitol, and dulcitol in pomegranate juices. The contents of D-sorbitol and dulcitol decreased with the fruit development, which was in contrast to the contents of sugar, probably due to the equilibrium conversion between sugars and sugar alcohols (Figure 1a). The 17 organic acids were succinic acid, citric acid, maleic acid, quinic acid, shikimic acid, L-ascorbate, L(-)-malic acid, L-(+)-tartaric acid, azelaic acid, adipic acid, glutaric acid, suberic acid, 2-hydroxybutanoic acid, D-glucuronic acid, gluconic acid, glutaric acid, and D-xylonic acid. Citric acid, D-xylonic acid, quinic acid, and L(-)-malic acid were the main organic acids in the juice of ripe fruits (Figure 1b).

Twenty essential amino acids were detected in the pomegranate juice. The decreasing amino acid contents during fruit development were in accordance with their corresponding organic acid contents. In the juices of ripe fruits, great amounts of L-Aspartic acid, L-Phenylalanine, L-Glutamine, and L-Tryptophan were accumulated in the cultivars from 'Tunisia' and great amounts of L-(+)-Lysine and L-Tryptophan were accumulated in the cultivars from 'Dabenzi' (Figure 1c).

A large number of alkaloids, including piperidine, betaine, and trigonelline, and small amounts of hordenine, camptothecin, and theobromine were detected in pomegranate juices. Camptothecin and theobromine were alkaloids reported in pomegranates for the first time. Except for isoquinoline, most alkaloids decreased during fruit development (Figure 1d).

It is reported that quinic acid act as a precursor for the biosynthesis of flavonoids and aromatic amino acids in plants [19]. Therefore, the large amount of quinic acid in pomegranate juices may be converted into rich flavonoids. We detected 23 flavones, 26 flavonols, 15 flavanones, 11 catechins and their derivatives, 10 anthocyanins, and 4 pro-anthocyanidins pomegranate juices (Figure 1). The main flavones were chrysoeriol O-acetylhexoside, chrysoeriol O-glucuronic acid, tricetin O-saccharic acid, tricetin 5-O-hexoside, and apigenin 5-O-glucoside. A large amount of quercetin was accumulated in the juices of early fruits, while great amounts of quercetin 4'-O-glucoside and quercetin 3-O-glucoside were accumulated in juices of ripe fruits, which indicated flavones glycosylation frequently occurring in the later development of fruits. In addition, as the core metabolite of flavanone biosynthesis, a large amount of naringenin existed in the glucosides of ripe fruits. Several flavonol contents decreased during fruit development, except for dihydromyricetin, myricetin 3-O-rhamnoside, and myricitrin in 'Dabenzi'.

Anthocyanins are one of the most important flavonoids that contribute to the color of fruits and flowers [20]. The increasing amount of anthocyanin leads to greater fruit pigmentation. In pomegranate juice, the major pigments are cyanidin, followed by pelargonin and delphinidin. Trace peonidin and rosinidin were detected in the pomegranate juices. Meanwhile, greater amounts of anthocyanidin, including cyanidin 3,5-O-diglucoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and pelargonidin 3-O-beta-D-glucoside, were detected in the juices of ripe fruits. We found that a great amount of anthocyanidin was accumulated in 'Dabenzi' than in 'Tunisia' (Figure 1h).

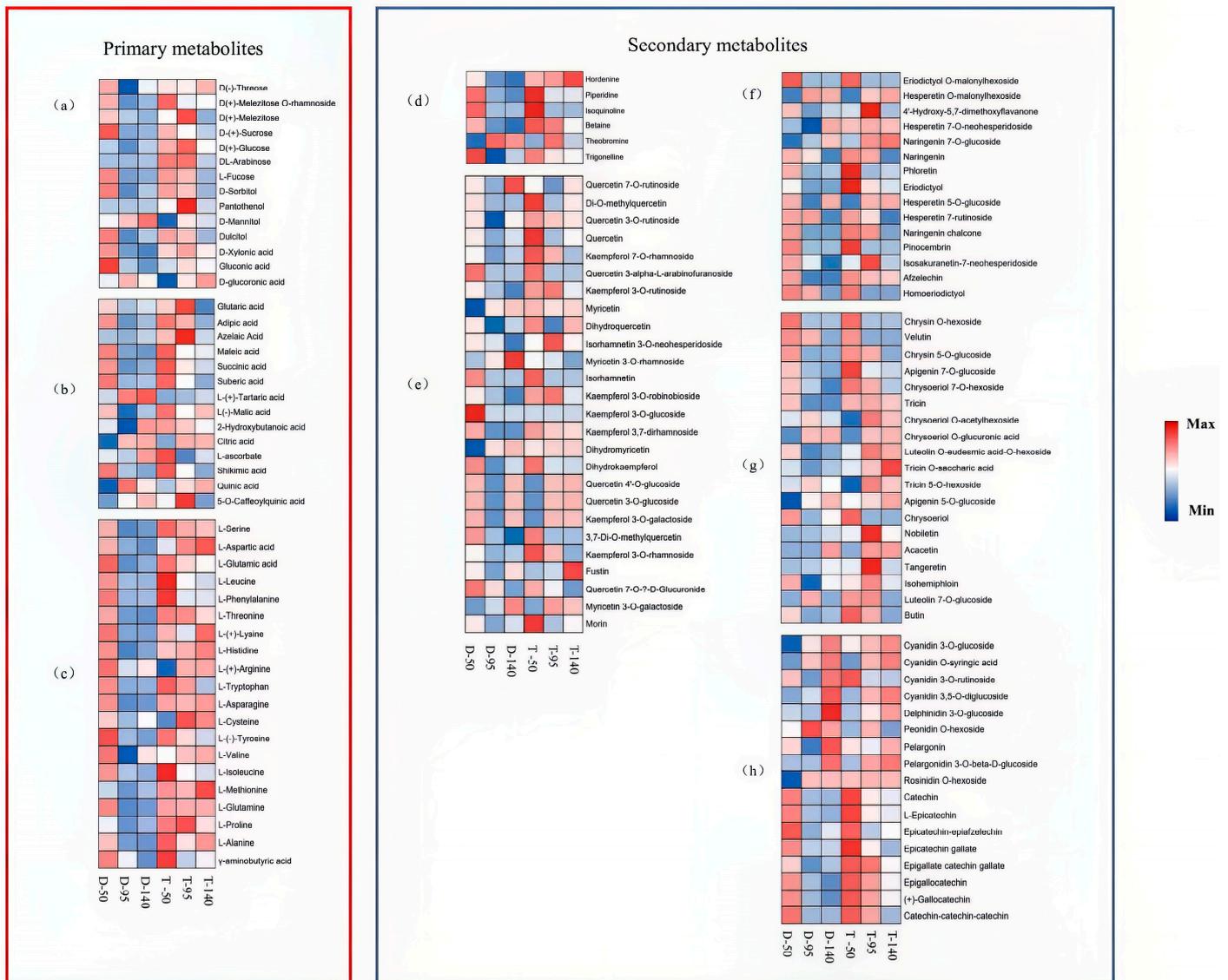


Figure 1. Heatmaps of relative content of metabolites in pomegranate juices. (a) Sugar and sugar alcohols; (b) organic acids; (c) amino acids; (d) alkaloids; (e) flavonols; (f) flavanones; (g) flavones; (h) flavonoids. The values are the means of three biological replicates. The letters D and T at the bottom stand for cultivar *P. granatum* ‘Denbenzi’ and *P. granatum* ‘Tunisia’. Numbers after D and T stand for days after flowering (DAF). The bar stands for the relative contents of metabolites. The data are the means of three replicates.

Catechin and its derivatives are another kind of flavonoid. They are derived from phenylpropanoid biosynthesis and endow fruits with a bitter and astringent taste. In pomegranate juices, we detected seven catechin derivatives, namely, epicatechin (EC), epicatechin-epiafzelechin (EC-E), epicatechingallate (ECG), epigallocatechingallate (EGCG), epigallocatechin (EGC), gallocatechin (GC), and catechin-catechin-catechin (CCC). Among them, EC, ECG, EGC, and GC were the main catechin derivatives in fruits at the early developmental stage, whereas EC, EGCC, EGC, and GC were the main catechin derivatives in the juices of ripe fruits. It is worth mentioning that the contents of catechin and its derivatives were higher in fruits at the early developmental stage (50 DAF) than in ripe fruits (Figure 1h), which was opposite to that of anthocyanin (Figure 2). The decreasing amounts of catechin and its derivatives in developing fruits meant a less bitter and astringent flavor, whereas increasing amounts of anthocyanin meant more pigments, which was in accordance with the favor we tasted and the color we perceived.

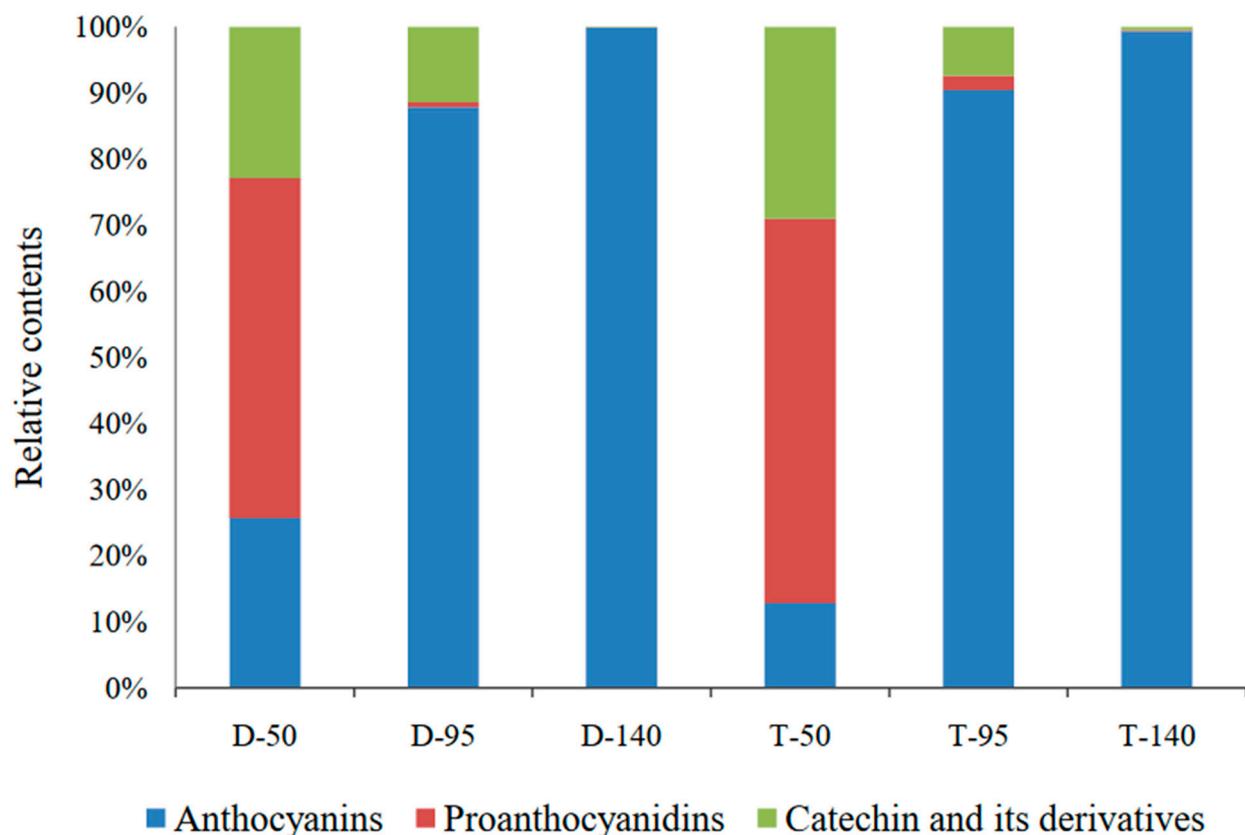


Figure 2. Relative contents of anthocyanins, proanthocyanidins, and catechin and its derivatives in pomegranate juices during fruit development.

3.3. Multivariate Analysis of Metabolic Profiling of Pomegranate Juices during Fruit Development

Complex primary and secondary metabolites constituted the metabolic profiling of pomegranate juice. The different constituents and contents of metabolites contributed to the distinguished flavors of fruits at different developmental stages. We found more downregulated metabolites in fruit juices from 140 DAF to 50 DAF than from 140 DAF to 95 DAF. Furthermore, the difference between 140 DAF and 50 DAF (Figure S1a,c) was larger than the difference between 140 DAF and 95 DAF (Figure S1b,d).

The number of differential metabolites detected in fruits at 50, 95, and 140 DAF between the two cultivars was 238, 216, and 148, respectively. The specific metabolites detected in the fruits at 50, 95, and 140 DAF between the two cultivars were 119, 74, and 21, respectively (Figure 3 left). When comparing the metabolic profiles between 50 and 140 DAF, we found 247 common and 33 special metabolites in ‘Dabenzi’ and 206 common and 60 special metabolites in ‘Tunisia’. This meant that a greater change in metabolite composition and content occurred in the juices during early fruit development (Figure 3 right). These changes were associated with the great transaction of fruit flavor at the early developmental stages, whereas slight changes at the later developmental stages indicated stable flavor characteristics. These results were in accordance with the quality attributes reflected by the chemical parameters (Table 1).

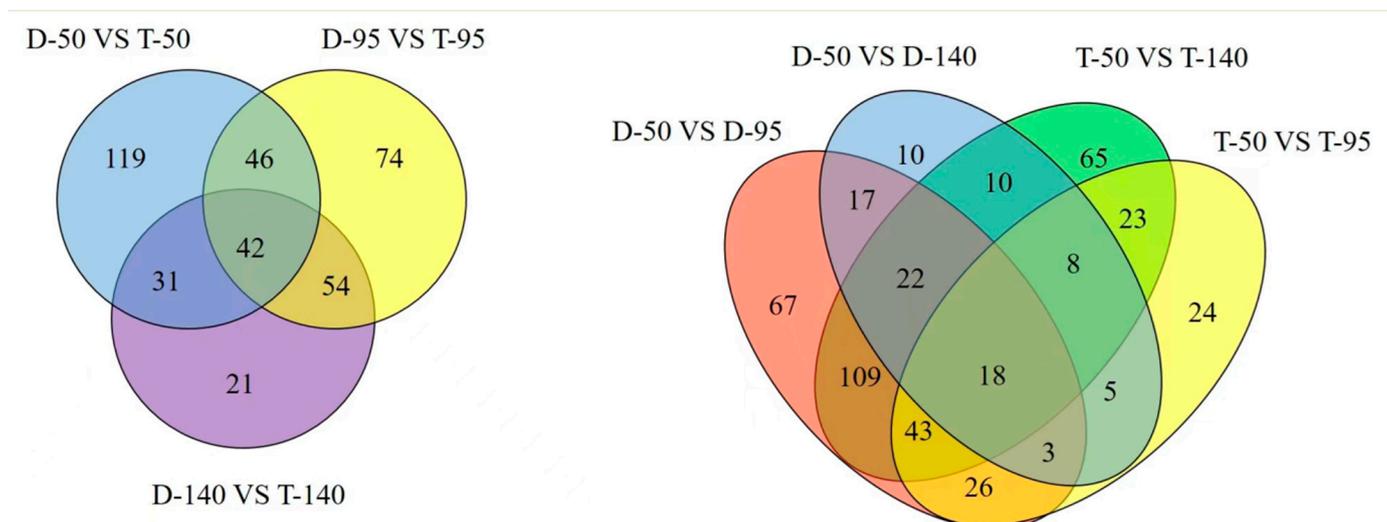


Figure 3. Venn diagram depicting the shared and specific metabolites in juices between the two cultivars at different developmental stages. Letter D and T at the bottom stand for cultivar *P. granatum* ‘Denbenzi’ and *P. granatum* ‘Tunisia’. Numbers after D and T stand for days after flowering (DAF).

3.4. Redirection of Flavonoid Metabolism and the Underlying Genetic Mechanism

To focus on the metabolites related to fruit development and flavor formation, we performed KEGG enrichment analysis on different metabolites. The main enriched KEGG terms for Dabenzi juices were biosynthesis of phenylpropanoid (map01061), flavonoid biosynthesis (map00941), and anthocyanin biosynthesis (map00941). The main enriched KEGG terms for ‘Tunisia’ juices were biosynthesis of phenylpropanoid (map01061), flavonoid biosynthesis (map00941), and phenylpropanoid biosynthesis (map00940). The common pathways related to both cultivars were enriched in flavonoid biosynthesis (map00941) and a branched pathway of phenylpropanoid biosynthesis (map00940) (Figure 4). To understand the genetic mechanism of flavonoid metabolism, transcriptome sequencing was conducted on the corresponding samples. There were 1637 and 2441 differentially expressed genes (DEGs) and 521 common genes for ‘Dabenzi’ and ‘Tunisia’, respectively, during fruit development (Figure S2a), which were enriched in metabolic pathway (map01100) and flavonoid biosynthesis (map00941) (Figure S2b). The enriched metabolic pathway of these DEGs was in accordance with the enriched metabolic pathway of different accumulated compounds. The high expression of *Pgr022711.1* encoding *PgrPAL* and *Pgr010283.1* encoding *PgrC4H* in Dabenzi at the early developmental stage contributed to p-coumaroyl-CoA production, providing sufficient precursors for the synthesis of flavonoids in ‘Dabenzi’. Chalcone isomerase (CHI) catalyzes the conversion of chalcone to naringenin, the core compound in the flavonoid biosynthetic pathway. The high expression of *Pgr025966.1* encoding CHI in ‘Tunisia’ at early development may lead to a greater accumulation of naringenin and a less accumulation of down-stream compounds (flavones, flavonols, and anthocyanin) in ripe fruits of ‘Tunisia’ due to the low expression of *Pgr013784.1*, *Pgr013787.1*, *Pgr013789.1* encoding *F3’H*, *Pgr013655.1* encoding *F3H*, and *Pgr026644.1* encoding *F3’5’H*. DFR and ANS were the key enzymes involved in anthocyanin and flavonol synthesis. The higher contents of anthocyanins and flavonols may be ascribed to the greater transcription accumulation of genes encoding DFR, ANS, and so on.

The decreased catechin and its derivative contents in juices of developing fruits may be due to the decreased transcript accumulation of *Pgr024128.1* encoding LAR and *Pgr017032.1* encoding ANR. Coloring anthocyanins derived from leucoanthocyanidin catalyzed by ANS were further glycosylated by anthocyanidin 3-O-glucosyltransferase (BZ1) and anthocyanidin 3-O-glucoside 5-O-glucosyltransferase (UGT75C1). The increasing transcript accumulation of *Pgr021399.1* encoding DFR and *Pgr017842.1* encoding ANS in developing fruits may have contributed to anthocyanin accumulation in the juices of ripe fruits. The

increasing transcript accumulation of *Pgr015322.1*, a gene encoding BZ1, may have contributed to the high accumulation of delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and pelargonidin 3-O-beta-D-glucoside, and the increasing transcript accumulation of *Pgr000447.1*, a gene encoding UGT75C1, may have contributed to the high accumulation of cyanidin 3,5-O-diglucoside. Hence, it is deduced that increased transcript accumulation of *Pgr021399.1*, and *Pgr017842.1*, and decreased transcript accumulation of *Pgr024128.1* played important roles in the metabolic direction of leucoanthocyanidins and determined the redirection of metabolic flux from catechin and its derivatives synthesis to anthocyanin synthesis in pomegranate juices (Figure 5).

Anthocyanin and catechin derivatives are flavonoids derived from leucoanthocyanins catalyzed by DFR, ANS, LAR, and ANR, respectively. Catechin and its derivatives can further polymerize into pro-anthocyanidins. The rhythmic change in proanthocyanins, anthocyanin, and catechin and its derivative contents indicated the metabolite trade-off of the flavonoid metabolic pathway during fruit development. The decreased accumulation of catechin, catechin derivatives, and their polymers was the result of upregulated anthocyanin biosynthesis (Figure 2). With respect to the metabolic flux, it implied that the leucoanthocyanin flowed into different branches.

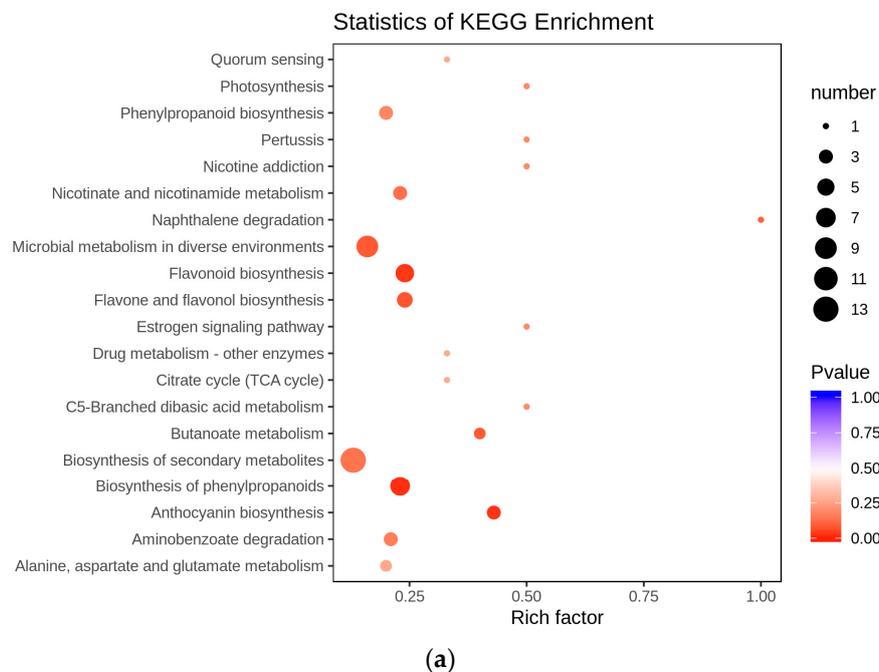


Figure 4. Cont.

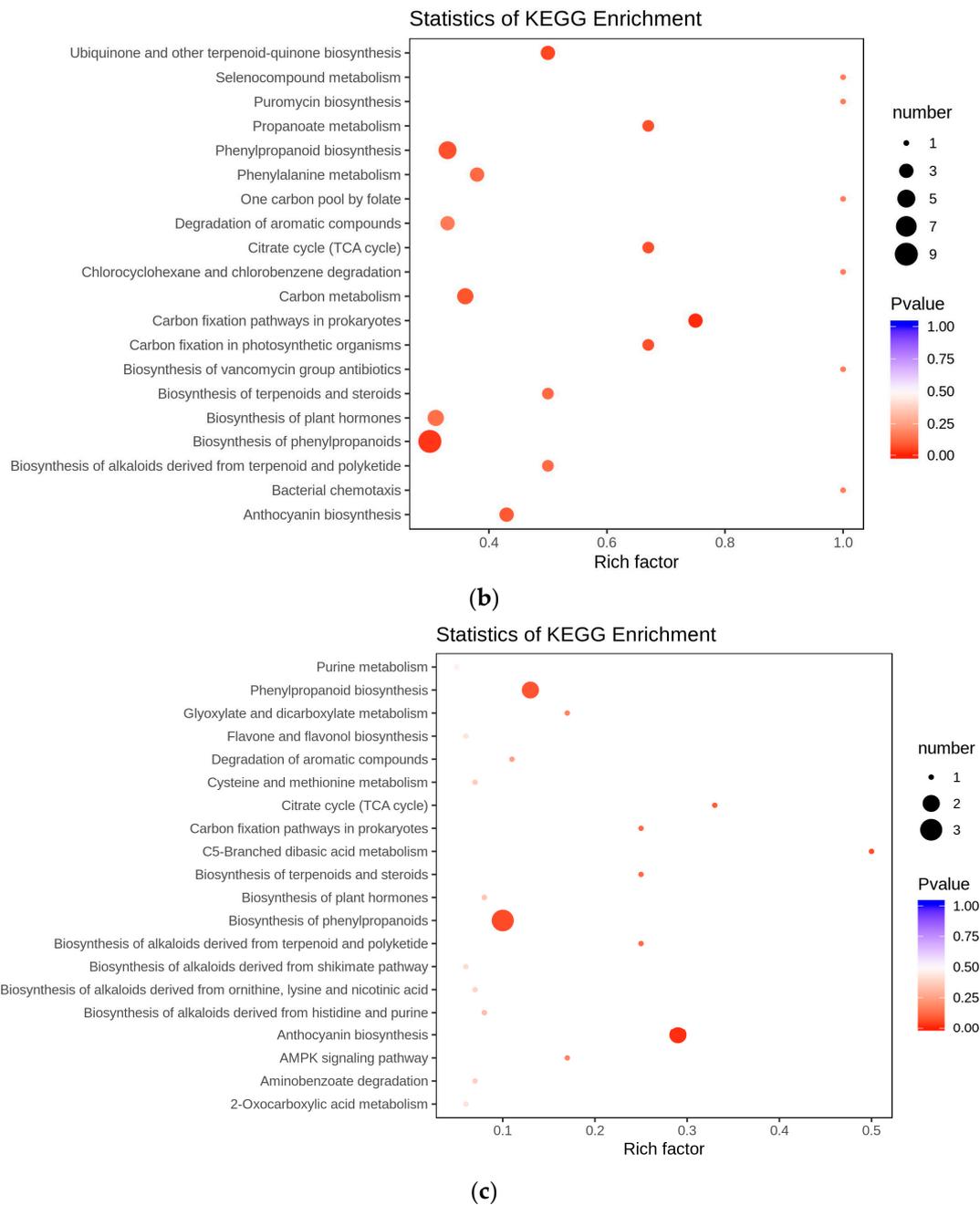


Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the metabolites involved in fruit development. (a) Enriched KEGG terms for 'Dabenzi'; (b) enriched KEGG terms for 'Tunisia'; (c) enriched KEGG terms shared by the two cultivars.

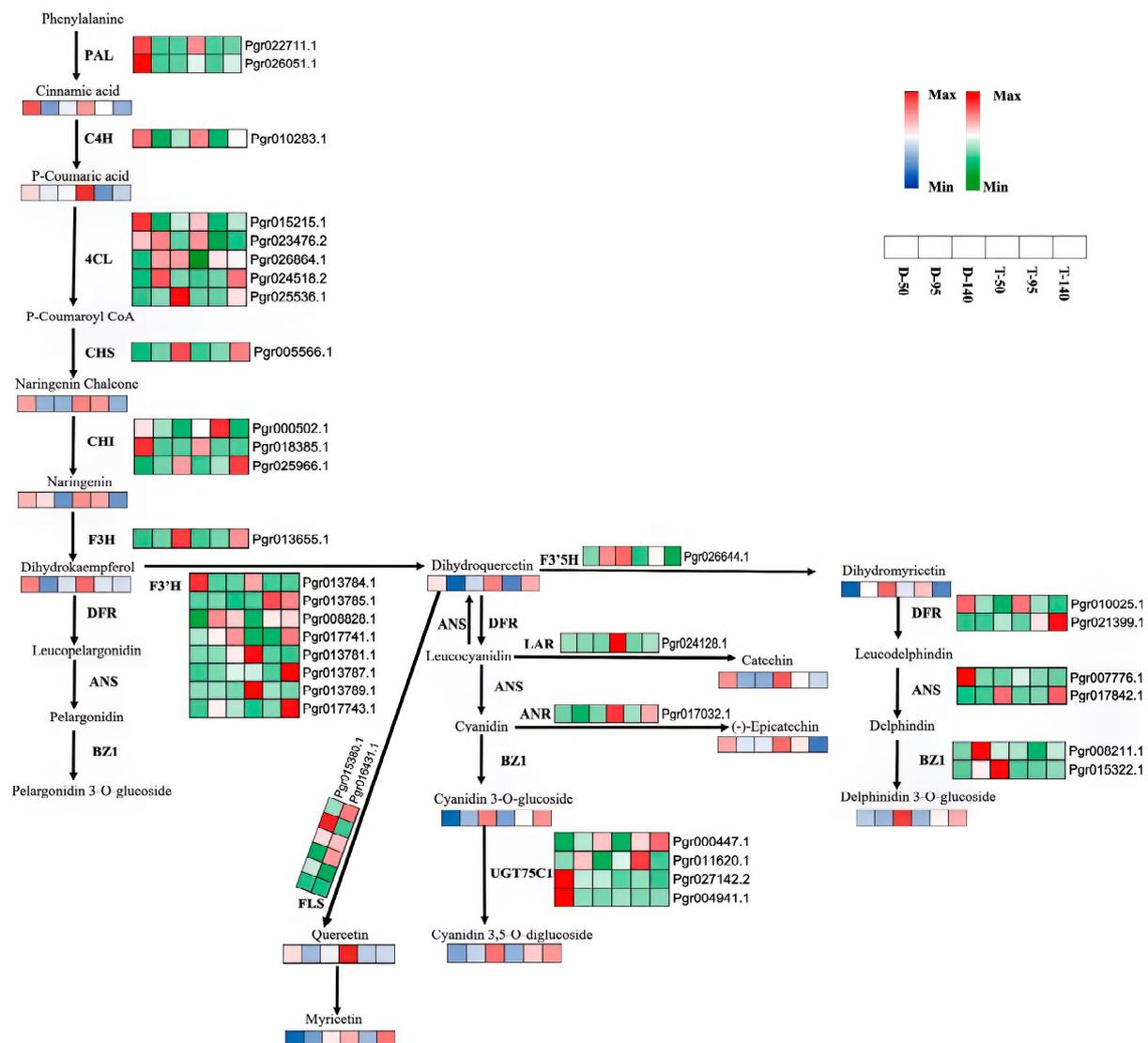


Figure 5. Biosynthetic pathway of flavonoids in pomegranate. Heatmap under metabolites were relative contents of metabolites in ‘Denbenzi’ and ‘Tunisia’ juices at 50, 95, and 140 DAF (days after flowering), respectively, from left to right. Heatmap beside the enzymes shows the relative expression levels of genes encoding enzymes in Denbenzi and Tunisia juices at 50, 95, and 140 DAF, respectively, from left to right. Letter D and T at the bottom stand for cultivar *P. granatum* ‘Denbenzi’ and *P. granatum* ‘Tunisia’. Numbers after D and T stand for the DAF. The values are the means of three biological replicates. Bars represent the relative contents of metabolites and the relative expression levels of genes encoding key enzymes. PAL: phenylalanine ammonia-lyase; C4H: cinnamic acid 4-hydroxylase; 4CL: 4-coumarate-CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3’H: flavanone 3’-hydroxylase; F3’5’H: flavanone 3’5’-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthesis; FLS: flavonol synthase; ANR: anthocyanidin reductase; LAR: leucoanthocyanidin reductase; BZ1: anthocyanidin 3-O-glucosyltransferase; UGT75C1: anthocyanidin 3-O-glucoside 5-O-glucosyltransferase.

4. Discussion

Pomegranate is widely cultivated in the world because of its rich nutrients and medicinal value [21]. A large number of metabolites, including sugars, organic acids, polyphenols including tannins and flavonoids, alkaloids, triterpenes steroids saccharides, coumarins, and lignans, were detected in pomegranate bark, root, leaf, and fruit by LC-MS or LC-MS/MS [22,23]. As we know, the coverage of metabolites is restricted by the methodologies

of sample preparation and the inherent sensitivity and specificity of the analytical technique employed [24]. Widely targeted metabolomics based on LC-MS/MS has been widely used in surveying the global metabolites of fruits. A comprehensive analysis of all the measurable analytes in a sample, including unknown chemicals, was conducted on several fruits, including strawberry [25], plum [26], mulberry [27], and pomegranate [28]. In this study, we detected 509 metabolites in pomegranate juices by performing a widely targeted metabolic analysis. In addition to the commonly detected compounds, metabolites such as peonidin, rosinidin, camptothecin, and obromine were detected in pomegranate for the first time. The comprehensive metabolic profiles of pomegranate juices can provide information for exploring pomegranate-based industrial products or foodstuffs.

Anthocyanin biosynthesis and catechin and its derivative biosynthesis are two branches of the flavonoid metabolic pathway; they share the central phenylpropanoid metabolic pathway [29]. Phenylpropanoid homeostasis among different branches is achieved via the regulation of MFR [30]. MFR can be regulated by genetic regulation and/on environmental stimuli. Genetic regulation occurs at transcriptional, post-transcriptional, post-translational, and epigenetic levels [31]. Multiple regulators, including structural genes encoding key enzymes and transcript factors, have been shown to regulate MFR [32,33]. In pomegranate juices, the higher expression of *Pgr005566.1* encoding CHS accompanied by the great accumulation of anthocyanin implied that CHS played important roles in pigmentation through the upstream regulatory system, similar to the coloration of the *Torenia hybrida* flower [34]. We found increased expression of the gene encoding ANS in pomegranate juices contributing to anthocyanin biosynthesis. While inactivation of ANS resulted in the blockage of anthocyanin biosynthesis and a shift in the accumulation of intermediate flavonoids in 'white' pomegranate [35]. The lack of ANS and DFR activity results in the loss of pigmentation in tobacco [36]. The high expression of *Pgr021399.1* encoding DFR, *Pgr017842.1* encoding ANS, *Pgr015322.1* encoding BZ1, and *Pgr000447.1* encoding UTG75C1 in pomegranate juices at later developmental stages indicated that *Pgr021399.1*, *Pgr017842.1*, *Pgr015322.1*, and *Pgr000447.1* played a key role in anthocyanin synthesis and pigment accumulation in the juices of ripe fruits. Catechins and their derivative biosynthesis were the subpathways of the flavonoid metabolism pathway. LAR catalyzed the conversion of leucoanthocyanidins into colored anthocyanidins. The decreased expression of *Pgr024128.1* encoding LAR may explain the decreased accumulation of catechins and their derivatives in fruit juices at the later developmental stages. The function of LAR in the synthesis of catechin and its derivatives was supported by increased proanthocyanidins in *TcLAR*-overexpressing transgenic tobacco. Overexpressing *TcLAR* in *Arabidopsis ldox* mutant also resulted in catechin and epicatechin elevated levels [37]. The transcription factor MYB alone, the co-expression of MYB and bHLH, and the formation of the MYB-bHLH-WD40 regulatory complex were sufficient to induce flavonoid biosynthesis in plants [36,38]. However, the regulatory effects of transcription factors on flavonoid biosynthesis in pomegranates are still unclear. It is considered that the evidence of structural genes and transcription factors in flavonoid biosynthesis depends on an efficient genetic transformation system, while the genetic transformation system of pomegranate has not been established in our lab or by others till now. So, efforts should be made to establish a genetic transformation system in the future. With the development of metabolic engineering and genetic engineering, genetic regulation of flavonoid metabolic flux using molecular design strategy has become promising.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9080881/s1>, Table S1: Metabolites detected in pomegranate juices. Figure S1: Partial least squares discrimination analysis (OPLS-DA) of metabolites identified from Dabenzi and Tunisia fruits at three different developmental stages. (a) D-50 vs. D-140; (b) D-95 VS D-140; (c) T-50 vs. T-140; (d) T-95 VS T-140. Figure S2: Venn diagram analysis and KEGG pathway enrichment of differentially expressed genes (DEGs) in pomegranate fruit at different developmental stages. (a) Venn diagram of DEGs; (b) enriched of DEGs.

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