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Transcriptome Analysis Identifies Genes Associated with Chlorogenic Acid Biosynthesis during Apple Fruit Development

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Abstract: As a predominant phenolic compound in apple fruits, chlorogenic acid (CGA) benefits human health due to its various antioxidant properties. However, little has been known regarding the molecular mechanism underlying the CGA accumulation in apple fruits. In this study, we measured the CGA content and relative enzymes' activities during fruit development in two different flesh-colored cultivars 'Huashuo' and 'Red Love'. The CGA content in both cultivars decreased sharply from 30 days after full bloom (DAFB) to 60 DAFB. Notably, the CGA content in fruit flesh was relatively higher than that in the peel. Further, the activities of C3H and HCT enzymes downstream of the CGA biosynthesis showed the similar changing trend as CGA content. Based on the transcriptome data of 'Huashuo' fruit at 30 DAFB and 60 DAFB, 23 differentially expressed CGA synthesis-related genes were screened. Gene expression analyses further showed that *MdHCT1/2/4/5/6* and *MdC3H1/2/3* were positively correlated to the variation of CGA content in two cultivars. These findings establish a theoretical foundation for further mechanism study on CGA biosynthesis and provide guidance for nutrient improvement in apple breeding programs.

Keywords: apple fruit; chlorogenic acid; transcriptome; gene expression

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1. Introduction

An apple is an economically important fruit with high nutrition that is widely cultivated around the world. In addition to a good taste, apple fruit is rich in phenolic compounds beneficial to human health [1]. Chlorogenic acid (CGA) is a predominant phenolic compound in apple fruits of most cultivars [2,3], and also the main phenolic compound in many other fruits, such as pears, peaches, strawberries, and blueberries [4–6]. In recent years, CGA has been shown to exhibit various antioxidant properties, including anti-diabetic, anti-microbial, anti-inflammatory, anti-hypertension, anti-obesity, cardioprotective properties, and neuroprotective effects [1,7–9]. Therefore, CGA is regarded as a health-promoting compound and has been widely used in different fields of food, medicine, and cosmetics.

The biosynthesis of CGA follows the phenylpropane metabolic pathway [10]. Phenylalanine is firstly dehydrogenated to form cinnamic acid under the action of phenylalanine ammonia-lyase (PAL) [11], and accordingly, there may be three following synthetic pathways. As shown, pathway 1 is based on the condensation reaction of quinic acid and caffeoyl-CoA, which is catalyzed by hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT/HQT). In pathway 2, the quinic acid and coumaroyl-CoA are catalyzed by Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT/HQT) to generate the p-coumaroyl-quinic acid, which then becomes CGA by hydroxylation under the presence of *p*-coumaroyl ester 3′-hydroxylase (C3′H). In pathway 3, CGA is produced from caffeoyl *D*-glucose and quinic acid, as catalyzed by the hydroxycinnamoyl *D*-glucose: quinate hydroxycinnamoyl transferase (HCGQT) [6,12]. The other key enzymes

that have been involved in the CGA biosynthesis include 4-coumarate-CoA ligase (4CL) and cinnamic acid 4-hydroxylase (C4H). Relative studies indicated that CGA is synthesized mainly through pathway 1 and pathway 2 [6], and HCT/HQT have been found to be rate-limiting enzymes [13]. Pathway 3 is only found in a few plant species such as sweet potatoes and tomato leaves [10,14].

The roles of critical genes involved in CGA accumulation have been widely studied in various plant species. For instance, the overexpression of *IbPAL1* in sweet potatoes increased the CGA levels in leaves [15]. In mulberries, the expression level of *MaHCT4* was positively correlated with the CGA abundance in leaves [16]. In pears, when *PbHCT4* was overexpressed in tobacco, the CGA content in transgenic plants was significantly higher than that in control plants [17]. In tomatoes, the overexpression or suppression of the *HQT* gene resulted in the up- or down-regulation of CGA levels accordingly [18]. However, limited studies on the molecular mechanism associated with CGA biosynthesis in apple fruits have been reported [1].

In this study, we measured the CGA contents and the enzyme activities related to CGA biosynthesis during growth of apple fruits in two different cultivars, i.e., a white-flesh cultivar 'Huashuo' and a red flesh cultivar 'Red Love'. Furthermore, the transcriptome sequencing and quantitative real-time PCR (qRT-PCR) were used to screen the differentially expressed CGA biosynthesis-related genes. This study is intended to reveal the molecular mechanism of CGA accumulation in apple fruits, which will be useful in future apple breeding programs that select cultivars with high CGA values.

2. Materials and Methods

2.1. Plant Materials

The apple fruits (*Malus domestica*) of two cultivars grown in the Science and Education Park of Henan Agricultural University were selected, including a white-flesh cultivar 'Huashuo' and a red-flesh cultivar 'Red Love'. 'Huashuo' fruits were collected at 30, 60, 90, 105, and 120 days after full bloom (DAFB), and 'Red Love' fruits were collected at 30, 60, 90, 105, 120, 135, and 150DAFB, respectively. At least 15 fruits were collected at each sampling time-point, and all samples were collected at a height of 1.7–1.9 m from the periphery of the canopy. The harvested fruits were brought back to the laboratory immediately. Then, the peels and fleshes were, separately, cut into small pieces, quickly frozen in liquid nitrogen, and then stored at –80 °C for further use. Each sample had three biological replicates.

2.2. Measurement of Chlorogenic Acid Content

The content of chlorogenic acid (CGA) was measured with a high-performance liquid chromatograph (HPLC) following the previously described method [19]. An amount of 0.25 g of frozen apple fruit tissues was crushed and the ground powder was homogenized with 5 mL methanol (containing 1% formic acid) through a 30 min ultrasonic treatment (60 kHz, 30 W). The homogenate was centrifuged at 6000 rpm for 5 min, and the supernatants were mixed again with methanol. This extraction process was repeated two times and the supernatants were combined. Subsequently, 1.5 mL of supernatant from the peel extract or 2.25 mL of supernatant from the flesh were evaporated on a rotary evaporator until there was no methanol phase, followed by dissolving in 150 µL methanol (containing 1% formic acid) and being filtered through a membrane. The resulting filtrate was subjected to HPLC analysis.

The CGA content was determined using a Waters 2695 HPLC System (Waters, America), equipped with a 2996 DAD detector, an ODS-C18 column (SunFire 5 µm, 250 mm × 4.6 mm). The HPLC analysis was performed in a mobile phase of 0.1% formic acid (solvent A) and 0.1% formic acid: acetonitrile (50:50, *v*/*v*, solvent B) using the following gradient system: 0–45 min, 23–50% B; 45–65 min, 50–80% B; 65–68 min, 80–100% B; 68–73 min, 100% B; 73–76 min, 100–23% B; 76–80 min, 23% B. The flow rate was 1 mL/min at 25 °C, and a volume of 10 µL samples was injected for HPLC analysis.

2.3. Determination of HCT and C3H Enzymatic Activities

HCT and C3H enzymes were assayed according to the previous methods [20]. An amount of 0.2 g of frozen apple peel or flesh tissues was crushed and homogenized with phosphate buffer saline (containing 0.05 mol/L Tris-HCl, pH 7.4; weight (g): volume (mL) = 1:4) in a 2 mL centrifuge tube. The homogenate was fully vortexed for 1 min, centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was used for the measurement of enzyme activities, using an HCT Elisa detection system kit and a C3H Elisa detection system kit of Kejing Biological Technology Co., Ltd. (Yancheng, China).

2.4. Transcriptome Sequencing

Transcriptomic sequencing was used to screen the differentially expressed genes related to CGA biosynthesis. Based on the changes of CGA content in the different stages of the two apple cultivars, the fruit flesh of 'Huashuo' apples at 30 and 60 DAFB with three biological replicates were used for transcriptome sequencing. Total RNA extraction, library construction, as well as RNA-seq were completed by Biomarker (Beijing, China). The libraries were sequenced on the Illumina NovaSeq 6000 platform.

2.5. RNA Extraction and cDNA Synthesis

Total RNA extraction from frozen fruit peel and flesh was performed according to the method described earlier [21]. The extracted total RNA was treated with HiScript® III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme) to remove contaminating gDNA. Subsequently, 1.0 μg RNA was used as template for cDNA synthesis with a Reverse Transcription System (Vazyme). At each sampling time-point, three biological replicates were used for RNA extraction.

2.6. Oligonucleotide Primers and Quantitative Real-Time PCR Analysis

Oligonucleotide primers were designed using Primer3 (version 0.4.0, https://bioinfo.ut.ee/primer3-0.4.0/ (accessed on 20 March 2021)). The gene specificity of primers was checked by a melting curve and the resequencing of the PCR product. The primer sequences are listed in Supplementary Table S1. To monitor mRNA abundance, we have chosen the apple Actin gene as a housekeeping gene [22]. Real-time PCR analysis was performed on the C1000 Touch Thermal Cycler instrument (Bio-Rad). The PCR reaction mixtures and the following program were based on our previous reports, with SYBRTM Select PCR Master Mix (Applied Biosystems) [23].

2.7. Statistical Analysis

Statistical significance of differences was analyzed using Microsoft Office Excel 2019. Figures were drawn with GraphPad Prism 8. The heatmap was constructed with TBtools. [24].

3. Results

3.1. Changes of Chlorogenic Acid Content during Fruit Development

The CGA content in apple fruits of the two cultivars, 'Huashuo' and 'Red Love', showed a similar decreasing trend during fruit development stages (Figure 1). Notably, the CGA content in the flesh at 30 DAFB was significantly higher than that of the peel for both cultivars. The content of CGA in the peel and flesh of 'Huashuo' apples reached the peak at 30 DAFB with a value of 16.66 μg/g and 271.93 μg/g, respectively. Similarly, the CGA contents in the peel and flesh of 'Red Love' apples also reached the peak at 30 DAFB with a value of 34.80 μg/g and 237.34 μg/g, respectively. Subsequently, the CGA content of the two cultivars showed a cliff-like decline at 30–60 DAFB, and then kept at basal levels until the fruits' mature stage. The appearance of the apple fruits of the two cultivars differs: the color of 'Huashuo' fruit peel is yellow and the color of 'Red Love' fruit peel is red.

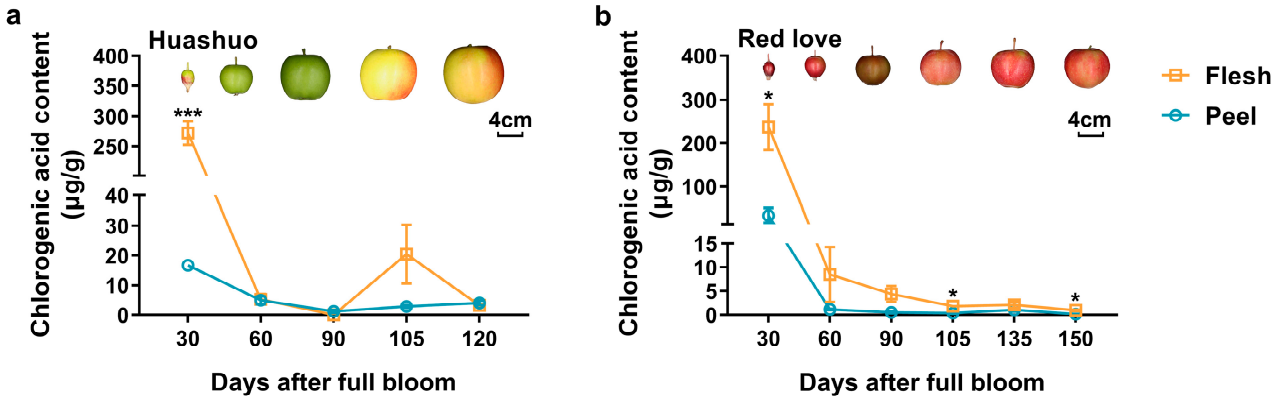

Figure 1. Changes of the CGA content during apple fruit development in the cultivars 'Huashuo' (**a**) and 'Red Love' (**b**). Apple fruit appearance for the cultivars 'Huashuo' and 'Red Love' at different stages are shown at the top of the figure (Scale bars = 4 cm). Error bars represent standard errors from three biological replicates. (* $p < 0.05$, *** $p < 0.001$).

3.2. Changes of HCT and C3H Enzymatic Activities during Apple Fruit Development

HCT activity in fruit flesh of 'Huashuo' cultivar decreased sharply from 30 DAFB to 60 DAFB, and then remained almost unchanged until the fruit became mature (Figure 2a). The levels of HCT activity in fruit flesh at 30 DAFB were significantly higher than those in the fruit peel. Furthermore, the changes in HCT activity during fruit development in 'Red Love' cultivar was similar to that in 'Huashuo', whereas the levels of HCT enzyme activity in 'Red Love' fruit were relatively lower than those in 'Huashuo' (Figure 2a).

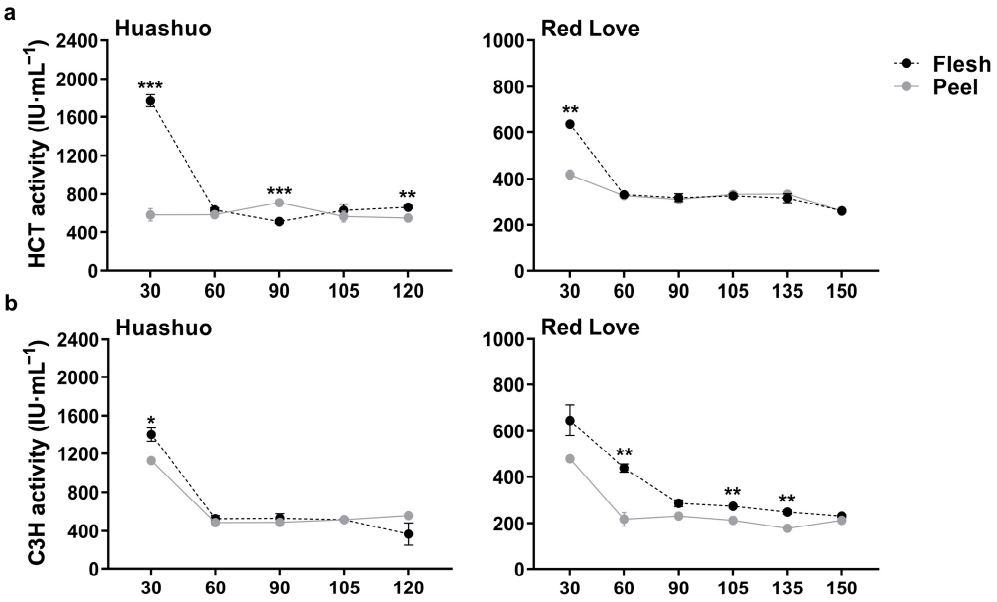

Figure 2. Changes in HCT (**a**) and C3H (**b**) enzyme activities during apple fruit development in cultivars 'Huashuo' and 'Red Love'. The black and gray circles represent the enzyme activity in the apple flesh and peel, respectively. Error bars represent standard errors from three replicates. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The C3H activity, another chlorogenic acid biosynthetic pathway-related enzyme, exhibited similar patterns in both cultivars. However, the difference of C3H enzyme activity at DAFB 30 between the fruit flesh and peel was narrowed (Figure 2b).

3.3. Transcriptomic Analysis of Differentially Expressed Genes

To obtain the key genes contributing to chlorogenic acid metabolism, six samples of fruit flesh from the 'Huashuo' cultivar at 30 and 60 DAFB were sent for RNA sequencing with three biological replicates, designated as HS-30d-A–C and HS-60d-A–C, respectively. The correlation analysis showed that three independent biological replicates were highly correlated (Figure 3a, r > 0.89). The differentially expressed genes (DEGs) were screened based on standard thresholds of the fold change $\geq$ 2.0 and false discovery rate (FDR) < 0.01. In total, 8589 DEGs were detected between HS-30d and HS-60d with 3250 up-regulated and 5339 down-regulated (Figure 3b,c). Based on the COG database, the DEGs were divided into 25 orthologous groups (Figure 3d), among which "general function prediction only" accounted for the largest (418; 11.33%), followed by "signal transduction mechanism" (403; 10.92%) and "transcription" (370; 10.02%). KEGG enrichment analysis showed that the DEGs between HS-30d and HS-60d were mainly enriched in 'plant hormone signal transduction', 'plant-pathogen interaction', and 'glycolysis/Gluconeogenesis' (Figure 3e).

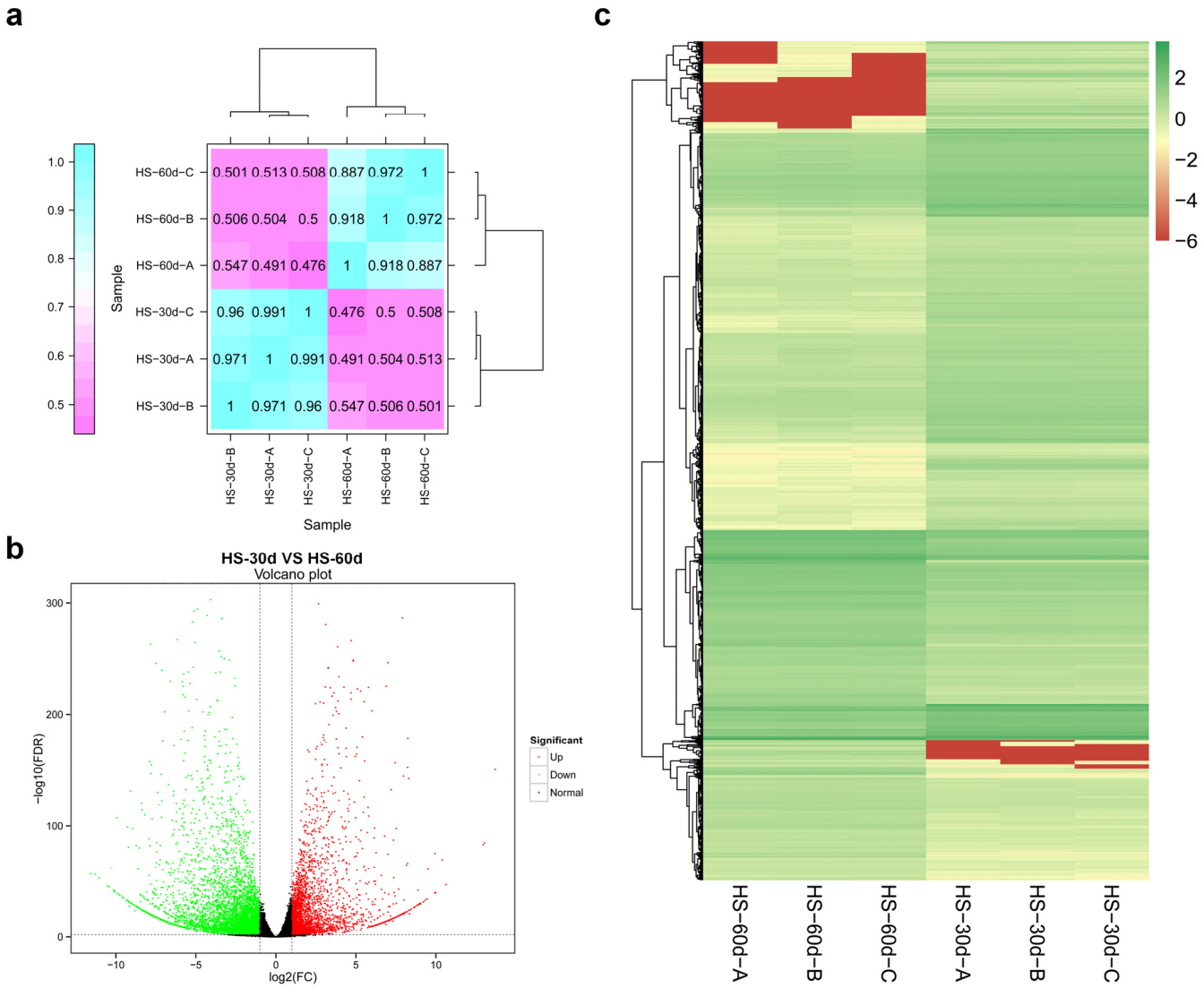

Figure 3. *Cont.*

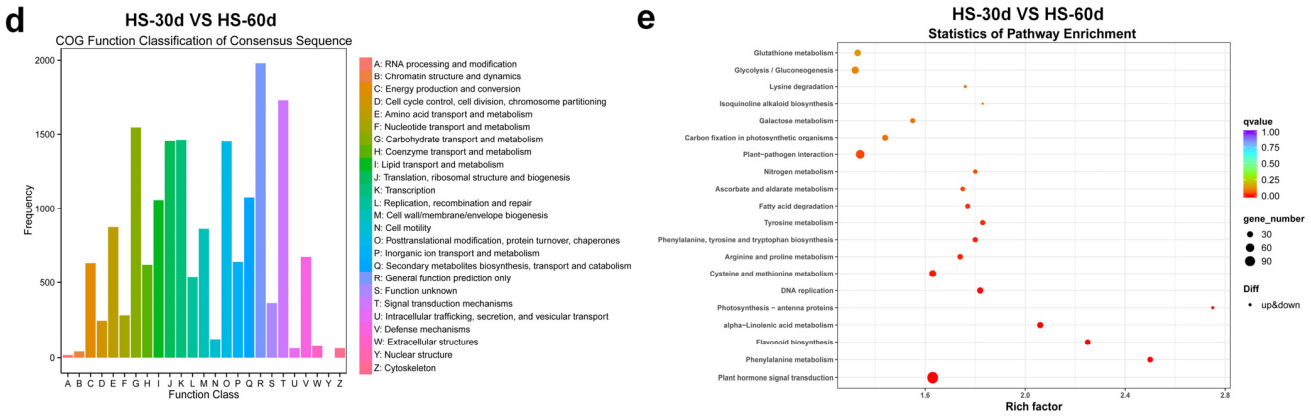

Figure 3. Transcriptome analysis of the DEGs in 'Huashuo' fruit flesh. (**a**) Correlation analysis between the six samples (HS-30d, HS-60d, with three replicates). HS-30d: Huashuo fruit at 30DAFB; HS-60d: Huashuo fruit at 60DAFB. (**b**) Volcano plot analysis of DEGs in HS-30d vs. HS-60d. (**c**) Heatmap of the expression profiles of the DEGs identified in HS-30d vs. HS-60d. (**d**) COG annotation classification of DEGs. (**e**) KEGG enrichment analysis of DEGs.

3.4. Analysis of Differentially Expressed Genes Associated with CGA Biosynthesis during Apple Fruit Development

Based on the obtained DEGs from RNA-seq, 23 differentially expressed CGA biosynthesis-associated genes were screened, including four *PAL* genes, four *C4H* genes, six *4CL* genes, six *HCT* genes, and three *C3H* genes (Figure 4). The transcript abundances of these CGA biosynthesis-associated genes were estimated by FPKM (fragments per kilobase of transcript per million fragments mapped) from RNA-seq data. Heatmaps were constructed using FPKM values to estimate the expression levels of these selected genes (Figure 4). Among the 23 differentially expressed CGA biosynthesis-associated genes, three *PAL* genes (MD01G1106900, MD04G1096200, MD12G1116700), three *C4H* genes (MD00G1221400, MD03G1050900, MD03G 1051000), four *4CL* genes (MD00G1033000, MD00G1033100, MD01G1236300, MD17G1229400), five *HCT* genes (MD09G1226600, MD16G1110600, MD16G1110700, MD17G1224900, MD17G12 25100), and three *C3H* genes (MD08G1242900, MD08G1243000, MD15G1436600) were highly expressed in fruits at 30 DAFB (with high CGA contents), which showed a significant positive correlation with CGA biosynthesis. Conversely, the other six genes, including one *PAL* gene (MD07G1172700), one *C4H* gene (MD11G1052900), two *4CL* genes (MD07G1309000, MD11G1145900), and one *HCT* gene (MD14G1155800), showed a significant negative correlation with CGA biosynthesis.

3.5. Validation of the Differentially Expressed CGA Biosynthesis-Associated Genes by qRT-PCR Analysis

In order to validate the accuracy of RNA-seq data, nine DEGs encoding the key enzymes associated with CGA biosynthesis were selected and analyzed by qRT-PCR in these two apple cultivars. In 'Huashuo' fruits, with the exception of *MdHCT3*, the expression levels of five other *HCT* genes (*MdHCT1/2/4/5/6*) and three *C3H* genes (*MdC3H1/2/3*) in both peel and flesh decreased during the fruit development, which showed a positive correlation with the CGA accumulation (Figure 5). Furthermore, the mRNA abundance of these genes in fruit flesh was significantly higher than that in the peel, which was consistent with the higher CGA content in the flesh than in the peel (Figures 1 and 5). Conversely, the expression level of *MdHCT3* increased gradually during fruit development, which was negatively correlated with the CGA accumulation. Generally, the expression patterns of these selected DEGs were similar to the RNA-seq data. Moreover, similar expression patterns of these selected nine DEGs were also verified during the fruit development of 'Red Love' apples (Figure 6), which further indicated that these genes may be key candidate genes involved in CGA biosynthesis.

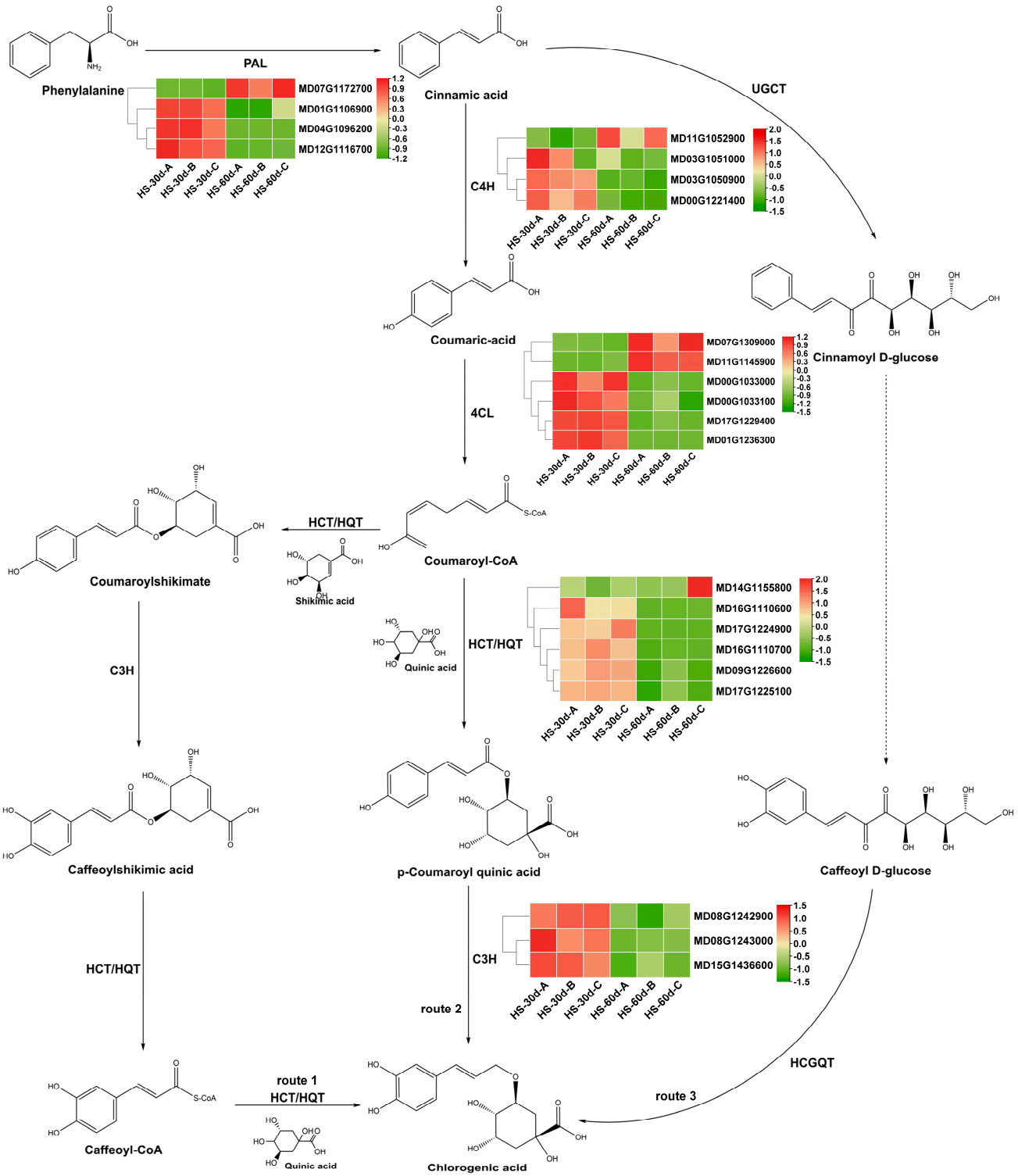

Figure 4. Schematic diagram of the CGA biosynthetic pathway and heatmaps of the expression patterns of DEGs associated with chlorogenic acid biosynthesis in 'Huashuo' fruits based on RNA-seq. PAL: phenylalanine ammonia-lyase; 4CL: 4-coumarate-CoA ligase; C4H: cinnamate 4-hydroxylase; C3H: *p*-coumarate 3-hydroxylase; HCT/HQT: hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; HCGQT: hydroxycinnamoyl *D*-glucose: quinate hydroxycinnamoyl transferase.

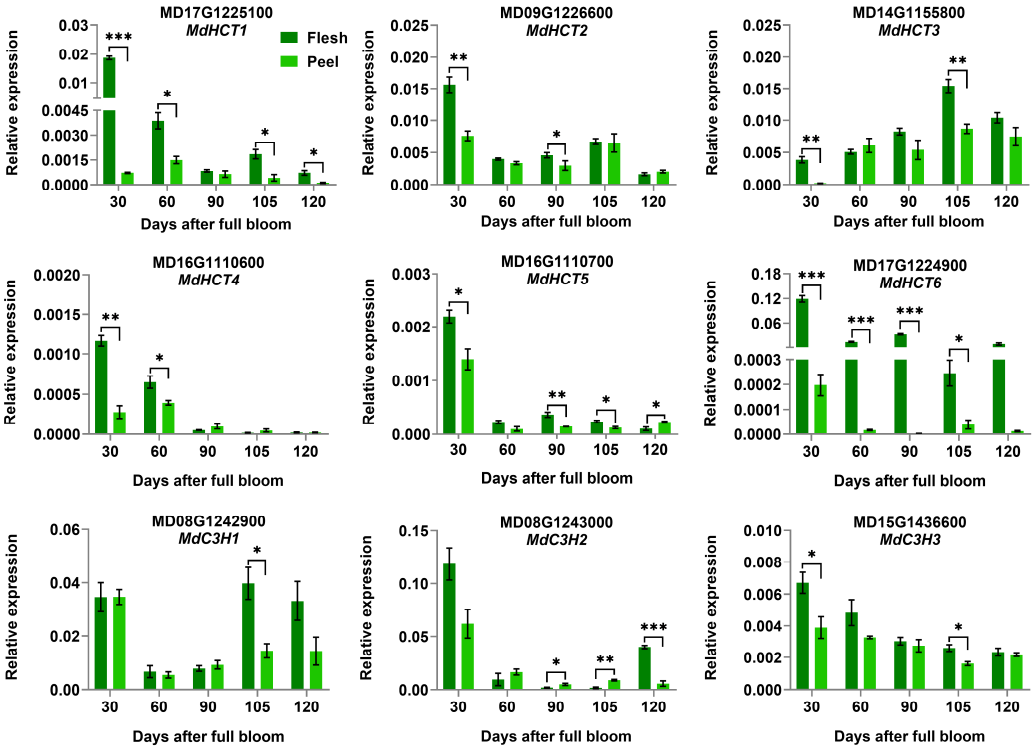

Figure 5. Quantitative expression analysis of candidate genes related to chlorogenic acid biosynthesis during fruit development in 'Huashuo' apples. Dark green columns and light green columns represent the expression levels of the genes in the flesh and the peel of the fruit, respectively. Error bars indicate standard errors from three biological replicates. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

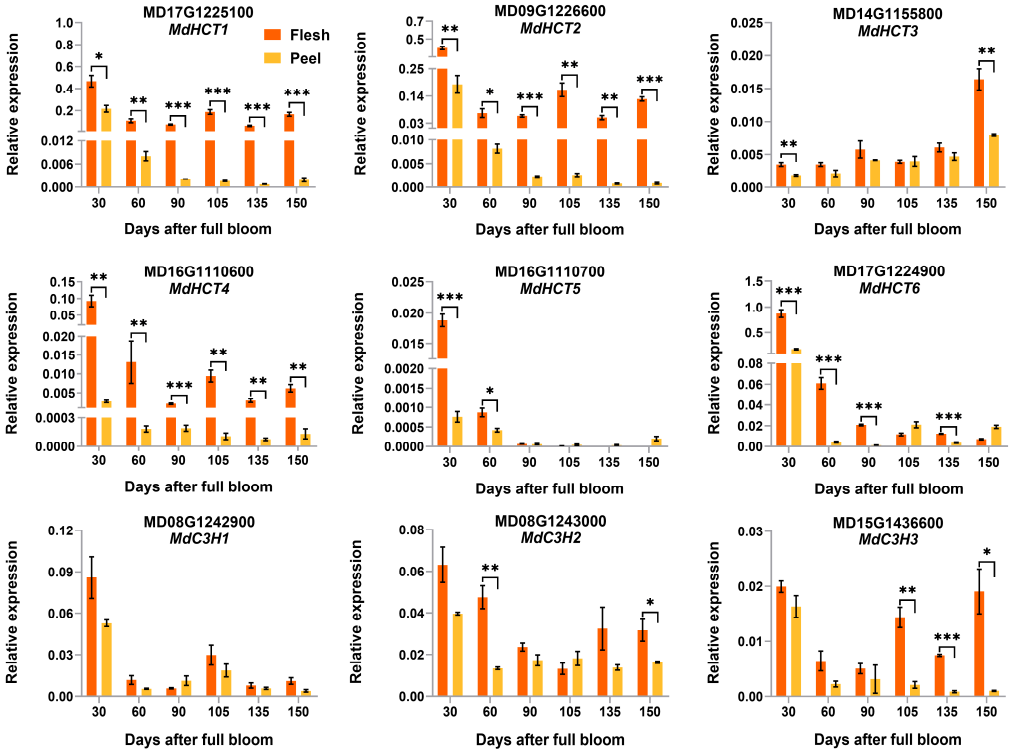

Figure 6. The relative expression levels of genes related to chlorogenic acid biosynthesis during fruit development in 'Red Love' apples. Dark orange columns and light orange columns represent the expression levels of the genes in the flesh and the peel of the fruit, respectively. Error bars represent standard errors from three biological replicates. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. Discussion

As one of the main phenolic compounds, CGA is abundant in various fruits, including apples, pears, peaches, and strawberries [1,4,6,25]. In this study, we found that the CGA content in both apple cultivars decreased gradually during the development, which was consistent with the previous report on apple fruits [1]. The decrease of CGA content during fruit development is probably caused by the enlargement of fruit volume, the hydrolysis of polyphenolic compound, or the decrease in the synthesis ability [26,27]. In addition, the CGA content in fruit flesh at the early stage of 'Huashuo' and 'Red Love' cultivars, and at the mature stage of the 'Red Love' cultivar, was significantly higher than that in the peel (Figure 1). Overall, our results were consistent with the previous studies (Hagen et al., 2007), and confirmed that the accumulation of CGAs was tissue-specific. Correspondingly, the activities of HCT and C3H enzymes downstream of the CGA biosynthesis in fruit flesh were also significantly higher than those in the peel (Figure 2), which indicated that HCT and C3H enzymes are closely associated with CGA biosynthesis.

Few studies on the molecular mechanism of CGA biosynthesis have been reported in apple fruits. Using RNA-seq, we identified 23 DEGs encoding five kinds of enzymes involved in the first and second pathways of CGA biosynthesis, including four *PAL* genes, four *C4H* genes, six *4CL* genes, six *HCT* genes, and three *C3H* genes. Similar to the case in many other plant species [28], no UGCT or HCGQT homolog was identified, indicating that the third pathway for CGA biosynthesis may not exist in apple fruit. PAL, C4H, and 4CL are key enzymes upstream of the phenylpropane metabolic pathway. They not only participate in the CGA biosynthesis, but also get involved in the synthesis of other secondary metabolites [29,30]. In sweet potato, overexpression of *IbPAL1* promoted CGA accumulation in leaves [15]. LmMYB15 can bind and activate the *4CL* promoter to promote CGA biosynthesis in *Lonicera macranthoides* [31]. In this study, the expression levels of three *PAL* DEGs (MD01G1106900, MD04G1096200, MD12G1116700), three *C4H* DEGs (MD03G1051000, MD03G1050900, MD00G1221400), and four *4CL* DEGs (MD00G1033000, MD00G1033100, MD17G1229400, MD01G1236300) positively corresponded with the CGA content (Figure 4), suggesting that these genes may play essential roles in CGA biosynthesis in apple fruit.

In addition, HCT/HQT and C3H are key enzymes downstream of the CGA biosynthesis pathway, with the C3H belonging to CYP98A subfamily and HCT belonging to the BAHD acyltransferase family [32,33]. Relative studies have shown that *HCT* or *C3H* genes are key genes in CGA biosynthesis in fruits. For instance, two *PpCYP45098A* genes and four *PpHCT* genes showed the same expression patterns as the CGA accumulation in peach fruit [25]. In strawberries, the blue light was able to co-upregulate CGA biosynthesis and *FvHCT* gene expression [4]. In pear fruit, the expression levels of *PpC3H* and *PpHCT1/3* were consistent with the variation in CGA content [34]. In our study, five *HCT* genes (*MdHCT1/2/4/5/6*) and three *C3H* genes (*MdC3H1/2/3*) showed relative higher expression levels at the early development stage, and in the fruit flesh than in the peel for both 'Huashuo' and 'Red Love' cultivars (Figures 5 and 6), which showed a positive correlation with the CGA accumulation in apple fruits. Among these genes, except for *MdHCT1* (previously designated as *MdHCT*) and *MdC3H1/2* [1], the other genes are new candidate genes associated with CGA biosynthesis in apple fruits.

5. Conclusions

In this study, we systematically evaluated the CGA content and relative enzyme activities during the development of 'Huashuo' and 'Red Love' apple fruits. The CGA content in both cultivars decreased sharply from 30 DAFB to 60 DAFB, and the CGA content in fruit flesh was relatively higher than that in peel. Correspondingly, the activities of C3H and HCT enzyme showed the similar changing trend as CGA content. By comparing the transcriptome data of DAFB 30 and DAFB 60 in 'Huashuo' apple, 23 DEGs associated with CGA biosynthesis were identified. The results of qRT-PCR showed that the expression patterns of *MdHCT1/2/4/5/6* and *MdC3H1/2/3* were positively correlated with the CGA

content during the development of apple fruits in both cultivars, indicating that these genes may play important roles in the CGA biosynthesis. These findings provide new data for the molecular mechanisms of CGA biosynthesis and guidance for future breeding of apple fruits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9020217/s1, Table S1: List of primers used for the qRT-PCR.

Author Contributions: Methodology, H.W. and Y.W.; formal analysis, W.Z., Y.G. and M.C.; investigation, H.W. and Y.W.; data curation, T.B., J.J., C.S. and S.S.; writing—original draft preparation, H.W.; writing—review and editing, X.Z. and M.W.; supervision, M.W.; funding acquisition, X.Z. and M.W. All authors have read and agreed to the published version of the manuscript.

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

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