Horticulture, Environment, and Biotechnology (2022) 63:403–411
https://doi.org/10.1007/s13580-021-00399-y

RESEARCH REPORT

Chlorogenic acid accumulation and related gene expression in peach fruit

Juan Yan[1] · Ziwen Su[1,2] · Shaolei Guo[1] · Minghao Zhang[3] · Binbin Zhang[1] · Zhixiang Cai[1] · Zhijun Shen[1] · Ruijuan Ma[1] · Mingliang Yu[1]

Received: 15 June 2021 / Revised: 18 September 2021 / Accepted: 20 September 2021 / Published online: 7 March 2022

Abstract
To reveal the molecular mechanism in the accumulation of chlorogenic acids (CGAs) in peach (*Prunus persica*) fruit during growth and development, CGA contents in the flesh of the three peach cultivars 'Ruiguang 18', 'Heiyoutao', and 'Beijingyixianhong' were determined. The expression levels of CGA metabolism-related genes were analyzed based on transcriptome data (RNA-seq). These candidate genes were then screened and real-time fluorescent quantitative PCR (qRT-PCR) was performed to verify their expression. The results showed that the content of total CGAs, 5-*O*-caffeoylquinic acid and 3-*O*-caffeoylquinic acid, in the flesh of 'Ruiguang 18' exhibited a decreasing trend during fruit development, and there was a great drop at maturity stage ($P < 0.05$). The three contents in 'Heiyoutao' increased first at stage S2 ($P < 0.05$) and then decreased significantly ($P < 0.05$). In 'Beijingyixianhong', they stayed stable in the early stages, then total CGAs and 3-*O*-caffeoylquinic acid decreased significantly at the maturity stage ($P < 0.05$). RNA-seq transcriptome data analysis and qRT-PCR expression analysis showed that the accumulation of CGAs in fruit flesh was mainly affected by the expression of *Prupe.3G100800* (*PpHCT*) and *Prupe.3G107300* (*Pp4CL*), and their expression levels were highly consistent with total CGA content. Thus, we concluded that *Prupe.3G100800* (*PpHCT*) and *Prupe.3G107300* (*Pp4CL*) are the key genes for CGAs synthesis in peach flesh.

Keywords *Prunus persica* (L.) · Chlorogenic acids · RNA-seq · Gene expression · HCT

1 Introduction

Chlorogenic acids (CGAs) have a variety of physiological functions such as free radical scavenging, anti-oxidation, and anti-inflammation (Abdelghafar et al. 2018). They have been proven to have preventive and therapeutic effects on type II diabetes, cardiovascular diseases, and metabolic syndrome (Naveed et al. 2018). They are also closely related to the biological and non-biological resistance of plants to low temperature, ultraviolet radiation, pests, and diseases and can effectively inhibit the infection and spread of various fungi and bacteria (Hammerschmidt 2014; Martinez et al. 2017).

The most abundant phenolic acids in peach fruit are CGAs, existing in two isomers, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid (Villarino et al. 2011). There are many tests and comparisons of the content of CGAs in the fruit of different peach cultivars (Chang et al. 2000; Tomas-Barberan et al. 2001; Yan et al. 2014a; Lu et al. 2017) and some preliminary studies on the accumulation of CGAs in different peach fruit development stages (Zhang et al. 2018a). Contents of CGAs in fruit flesh of different peach cultivars varied from a few mg/kg FW to a few hundred mg/kg FW (Yan et al. 2014a). In some cultivars, CGAs dropped as the fruit ripened (Zhang et al. 2018a). Some cultivars have fluctuant CGA content in their fruit. For example, CGAs in 'Redhaven' reached the peak concentration at 94 days after full bloom (DAFB), decreased at 100 DAFB, and increased

Communicated by Heakeun Yun.

Juan Yan and Ziwen Su have contributed equally to this work.

✉ Mingliang Yu
mly1008@aliyun.com

[1] Institute of Pomology, Jiangsu Academy of Agricultural Sciences/Jiangsu Key Laboratory for Horticultural Crop Genetic Improvement, Nanjing 210014, China

[2] College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

[3] Forest and Fruit Tree Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai 201403, China

again at fruit ripening (Orazem et al. 2013). There have been many reports on the functions of CGAs (Villarino et al. 2011; Zhang et al. 2018b; Jiao et al. 2018; Vitus et al. 2019). For instance, Zhang et al. (2018b) confirmed that the main antioxidant ingredient in peach fruit was 5-*O*-caffeoylquinic acid rather than other phenolic substances such as catechins and ferulic acid. Peach fruits with higher CGA content show a higher resistance against pathogens causing brown rot (Villarino et al. 2011; Vitus et al. 2019). In addition, CGAs could inhibit the expansion of penicillium by activating the jasmonic acid signaling pathway to prevent the spoilage of peach fruit (Jiao et al. 2018). It can be concluded that the content of CGAs is closely associated with the quality of peach fruit. Therefore, a biosynthesis study of CGAs can provide some guidance for the improvement of peach nutrition and resistance to diseases.

The metabolism pathway of CGAs is unclear even in model plants like *Arabidopsis thaliana*. No research on how many pathways there are in peach or which one is the main pathway has been done so far. CGAs are formed by trans-hydroxycinnamic acid (such as coumarin, caffeic acid, ferulic acid, etc.) and quinic acid, existing in various structural forms such as derivatives and isomers (Jaiswal et al. 2014). Among them, most attention has been paid to caffeoylquinic acid because of its wide existence in plants. CGAs are synthesized via phenylpropanoid metabolism, and three possible pathways have been proposed, as shown in Fig. 1: (1) the trans-esterification of caffeoyl-CoA and quinic acid via hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase (HQT) activity; (2) the hydroxylation of p-coumaroyl quinate to CGA; and (3) the hydroxylation of p-coumaroyl shikimate to caffeoyl shikimic acid, which is then converted to caffeoyl-CoA, a substrate of hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase HCT. Hence, phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-hydroxycinnamoyl-CoA ligase (4CL), p-coumaroyl ester 3'-hydroxylase (C3′H), and HCT/HQT are important enzymes related to CGA biosynthesis (Naveed et al. 2018; Comino et al. 2007, 2009; Sonnante et al. 2010; Mudau et al. 2018). Their activities and functions are different among *Arabidopsis thaliana*, artichoke, tobacco, tomato, and honeysuckle (Mudau et al. 2018; Chen et al. 2017; Niggeweg et al. 2004; Sonnante et al. 2010). Although CGAs were found to exist widely in apples, pears, and peaches and their contents were high (Awad and Jager 2001; Cui et al. 2005; Yan et al. 2014b), there are few studies on the regulation of CGA synthesis in fruit, except for a report on pear (He et al. 2017). The study of CGA metabolism and gene expression regulation of related enzymes during peach fruit development is of great significance to reveal the mechanism of CGA metabolism.

We found that some red flesh peach cultivars not only had much higher CGA content than those with white and yellow flesh but also showed differences in the accumulation of CGAs during fruit ripening (Zhang et al. 2018a). Based on this finding, one yellow flesh peach cultivar 'Ruiguang 18' and two red flesh peach cultivars 'Heiyoutao' and 'Beijingyixianhong' were chosen as test materials to determine the content of CGAs in their flesh during the fruit development stages and analyze the differences in the expression of genes related to CGA metabolism so that the key genes regulating CGA metabolism could be found.

2 Materials and methods

2.1 Plant material

The experiment was conducted at the National Peach Fruit Germplasm Repository of Nanjing (32° 2′ N, 118° 52′ E, 11 m above sea level) in 2020. Mature trees of peach cultivars 'Ruiguang 18', 'Heiyoutao', and 'Beijingyixianhong' were selected as the test materials. The trees were of natural open center shape, with a row spacing of 5 m × 3 m, and the conventional cultivation measures were taken. Fruit development of the three cultivars can be divided into four stages (pit hardening, second exponential growth, enlargement, and maturity). The maturity stage refers to the time when

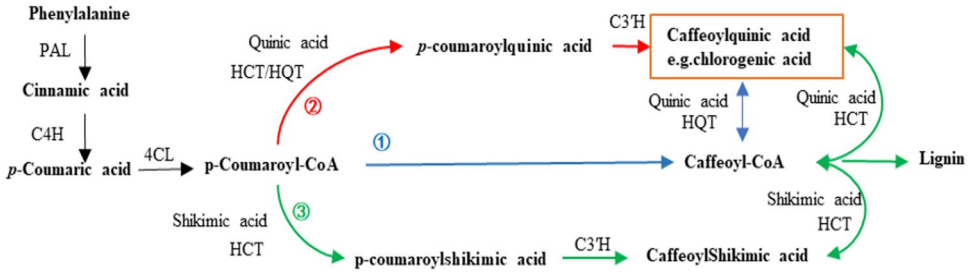

Fig. 1 A simplified diagram of enzymes and major products in the synthesis of CGAs in plants (Naveed et al. 2018; Comino et al. 2009). Enzymes involved in this pathway are as follows: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase; HQT, hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase; C3'H, p-coumaroyl ester 3'-hydroxylase

the background color of the fruit is changed. The sampling time was strictly chosen (at the beginning of each stage) according to the growth and development curve of peach fruit. Sampling was conducted according to the method used by Yan et al. (2018) and Lombardo et al. (2011). Fruits of 'Ruiguang 18' were harvested at 45, 78, 93, and 101 DAFB; 'Heiyoutao' at 50, 95, 109, and 126 DAFB; and 'Beijingyixianhong' at 40, 72, 85, and 92 DAFB. Ten disease-and-pest-free peaches with uniform size and maturity from two trees of each cultivar were collected. They were put in the icebox, quickly brought to the laboratory, and peeled with a peeler. Flesh on both sides of the fruit suture were chopped, mixed well, frozen immediately in liquid nitrogen, and stored at − 80 °C. Procedures were conducted in triplicates, and 30 fruits of each cultivar were collected in total.

2.2 Extraction and determination of CGAs

Ground frozen fruit tissues (0.5 g) were homogenized in 2 mL methanol solution containing 0.1% (v/v) H_3PO_4 and incubated in an ultrasonic oscillator for 10 min. After centrifugation at 10,000 rpm for 10 min at 4 °C, the supernatant was collected and filtered through a 0.22-mm organic membrane, and the filtrate was used to determine CGAs. An Agilent ZORBAX SB-C18 column (5 μm, 250 mm × 4.6 mm) was used in an Agilent 1260 Infinity II HPLC System (Agilent, USA) equipped with a DAD detector at 30 °C, and elution was performed with a mobile phase gradient (A: methanol solution containing 0.1% H_3PO_4, v/v, B: 0.1% H_3PO_4 water solution) at a flow rate of 1.0 mL/min. The gradient for buffer B was 95–29% for 22 min, 29–95% for 8 min, and 95% for 5 min. Dose-dependent calibration curves of 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid standards (Sigma-Aldrich Corporation, St. Louis, USA) and internal standards were used to determine the concentrations of the components.

2.3 Transcriptome sequencing

The Plant RNA Extraction Kit (Bio Te Ke, Beijing) was used to extract total RNA from peach flesh, and a NanoDrop ultra-micro spectrophotometer (Thermo Fisher Scientific, USA), Qubit2.0 fluorometer (Invitrogen, USA), and Agilent 2100 bioanalyzer (Agilent Technologies, China) were used to detect the purity, concentration, integrity, and other parameters of RNA samples. The library construction and sequencing were performed by Nanjing Genepioneer Biotechnologies Co., Ltd. with transcriptome sequencing on the Illumina high-throughput sequencing platform (HiSeq/MiSeq) using Peach Genome v2.0 as the reference genome (Jung et al. 2019).

2.4 The search for genes related to CGA metabolism

The amino acid sequences of PAL, C4H, 4CL, HQT, C3′H, and HCT gene families related to CGA metabolism in species such as *Arabidopsis thaliana*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Pyrus bretschneideri*, and *Coffea canephora* were found in the NCBI gene database, and similar peach genes were searched via BLAST. The peach genes found were compared with the genes in the transcriptome data, using the FPKM method (fragments per kilobase per million fragments) to calculate the gene expression level (Trapnell et al. 2010; Florea et al. 2013). The candidate genes related to CGA metabolism were obtained by comparing and analyzing the correlation with the accumulation trend of CGAs in the peach cultivars based on the changes of the FPKM value of each gene.

2.5 qRT-PCR analysis of candidate genes

Specific primers for candidate genes were designed using Primer 5.0 to perform real-time fluorescence quantitative analysis. The plant RNA kit (Bio Te Ke, Beijing, China) was used to extract total RNA from peach fruit samples used for expression verification, and reverse transcription was performed in a Goldenstar RT6 cDNA Synthesis Kit Ver 2 (Qingke Gold, Nanjing, China). The cDNA product obtained by reverse transcription was appropriately diluted and used as a qPCR template, and amplified with 2 × T5 Fast qPCR Mix (SYBR Green I) (Qingke, Nanjing, China). The reaction system (20 μL) included 0.8 μL of the upstream and downstream primers, 1 μL of the cDNA template, 7.4 μL of ddH_2O, and 10 μL 2 × T5 Fast qPCR Mix (SYBR Green I). The fluorescence quantitative PCR instrument FQD-96A (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China) was used for qPCR. Reaction conditions were as follows: pre-denaturation stage under 95 °C for 60 s; cycle stage under 95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s, 40 cycles; dissolution stage under 95 °C for 5 s, 60 °C for 60 s, 95 °C and 0.11 °C /s increasing, 50 °C for 30 s. TEF2 was used as the reference gene in gene expression analysis (Tong et al. 2009), and the $2^{-\Delta\Delta CT}$ method was used to calculate expression difference based on the Ct value with the amplification efficiency of 100% (Livak and Schmittgen 2001). Results were the average of three biological replicates ± standard deviation (SD).

2.6 Statistical analysis

Data processing, significant difference analysis, and correlation analysis were done using Excel 2003 and SPSS 16.0. One-way analysis of variance and the least significant difference method (LSD) were used for the significance analysis between the means, and the significant difference was at

5% level. The Pearson correlation method was used for the correlation analysis between CGA content and the FPKM value of each gene.

3 Results

3.1 Variation in CGA content during fruit development

It can be seen from Fig. 2 that the changes in CGA content in the flesh of the three peach cultivars were different. In the fruit of 'Beijingyixianhong', the contents of total CGAs and 3-*O*-caffeoylquinic acid had a similar increasing trend during fruit development (40DAFB-85DAFB) with no significant difference, and both significantly decreased at the fruit mature stage (85-92DAFB), while 5-*O*-caffeoylquinic acid decreased gradually with the fruit development with no significant difference. The content of total CGAs, 5-*O*-caffeoylquinic acid, and 3-*O*-caffeoylquinic acid in the flesh of 'Ruiguang 18' exhibited a decreasing trend during the fruit development and significantly decreased at the fruit mature stage ($P < 0.05$). The three contents in 'Heiyoutao' increased at 95 DAFB ($P < 0.05$) and then decreased significantly ($P < 0.05$). The proportions of 5-*O*-caffeoylquinic acid to 3-*O*-caffeoylquinic acid in the three cultivars were found to be different, in which the proportion in 'Ruiguang 18' was higher and much lower in 'Heiyoutao' and 'Beijingyixianhong'.

3.2 Screening of CGA metabolism-associated genes in peach fruit

Sixteen peach genes with similar amino acid sequences to the *PAL*, *C4H*, *4CL*, *HQT*, *C3'H*, and *HCT* gene families related to the CGA metabolism of other plants, including *Pyrus bretschneideri*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Cynara cardunculus*, *Lycopersicum esculentum*, and *Coffea canephora*, were screened using the NCBI gene database, in which there was one PAL-related gene (*Prupe.6G235400*), one C4H-related gene (*Prupe.6G040400*), three 4CL-related genes (*Prupe.1G087900*, *Prupe.3G107300*, and *Prupe.6G109000*), two C3'H-related genes (*Prupe.1G580500* and *Prupe.1G580200*), nine HCT-related genes (*Prupe.3G100800*, *Prupe.3G101000*, *Prupe.3G101400*, *Prupe.3G101500*, *Prupe.3G101600*, *Prupe.3G101700*, *Prupe.3G101800*, *Prupe.31900*, *Prupe.3G101800*, and *Prupe.6G286700*). No genes with high similarity to the *HQT* sequence were found in peach.

Transcriptome analysis showed that 4 *HCT* genes (*Prupe.3G101000*, *Prupe.3G101400*, *Prupe.3G101500*, and *Prupe.6G286700*) of the 16 genes had an expression level of 0. The FPKM value of the other 12 genes was analyzed by heat map, and the correlation analysis of those genes and total CGAs is shown in Fig. 3 and Table 1. During the development of peach fruit, the total amount of CGAs in the flesh of 'Ruiguang 18' was significantly positively correlated with the expression of *Prupe.1G087900* (*4CL*), *Prupe.3G107300* (*4CL*), and *Prupe.3G100800* (*HCT*), while it was significantly positively correlated with the expression of *Prupe.3G107300* (*4CL*), *Prupe.1G580500* (*C3'H*), *Prupe.3G100800* (*HCT*), and *Prupe.3G101900* (*HCT*) in 'Heiyoutao'. The total CGA content in the flesh of 'Beijingyixianhong' was significantly positively correlated with *Prupe.3G107300* (*4CL*) and *Prupe.3G100800* (*HCT*). Therefore, *Prupe.1G087900* (*4CL*), *Prupe.3G107300* (*4CL*), *Prupe.3G100800* (*HCT*), *Prupe.1G580500* (*C3'H*), and *Prupe.3G101900* (*HCT*) were screened as the candidate genes related to CGA metabolism in peach fruit.

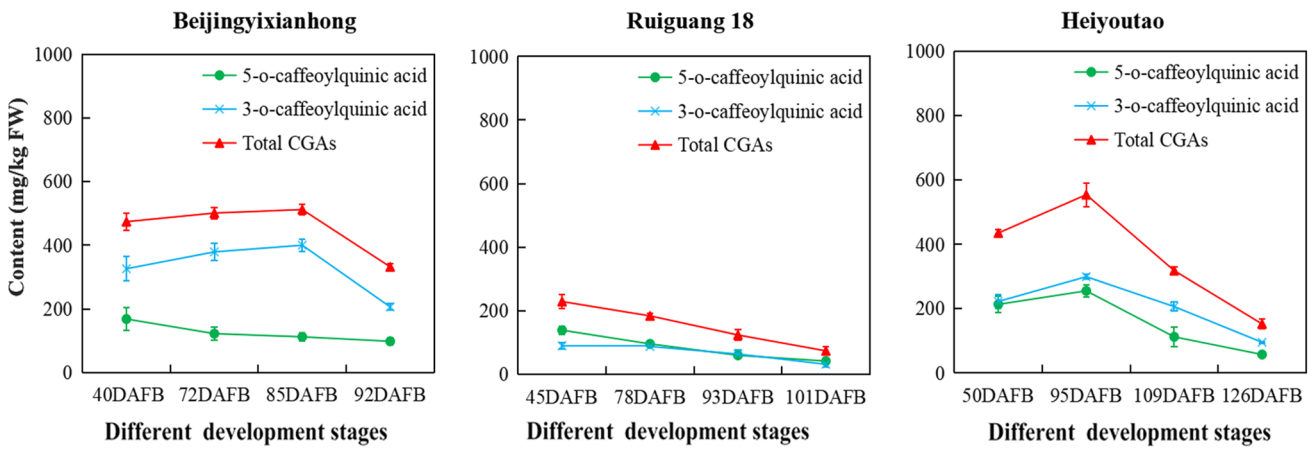

Fig. 2 Changes in CGA contents in peach flesh at different fruit development stages

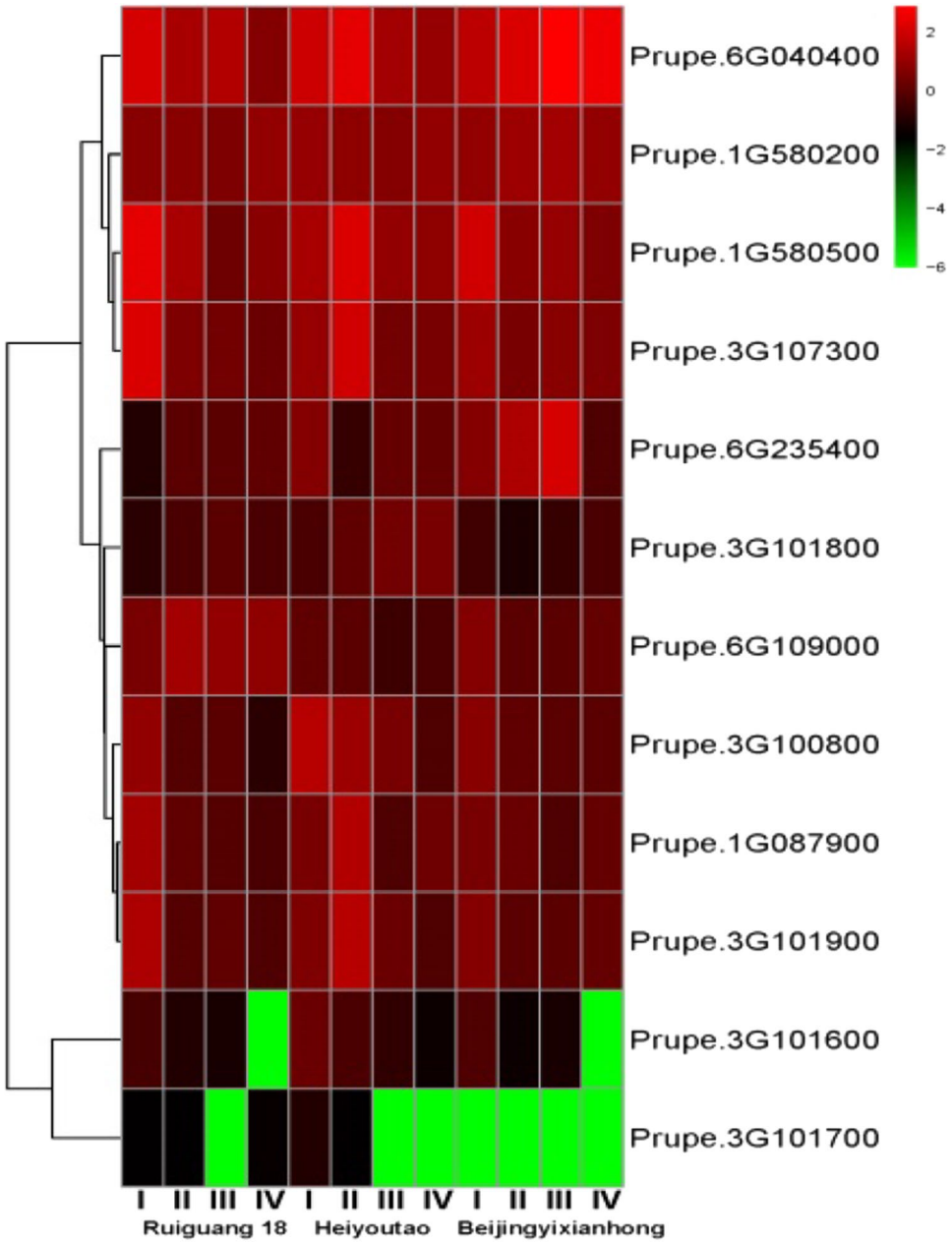

Fig. 3 Heat map of the expression of 12 CGA metabolism-related enzyme genes in peach flesh

3.3 Quantitative analysis of candidate genes expression

Specific primers for the five selected candidate genes (*Prupe.1G087900* (*4CL*), *Prupe.3G107300* (*4CL*), *Prupe.1G580500* (*C3'H*), *Prupe.3G100800* (*HCT*), and *Prupe.3G101900* (*HCT*)) are listed in Table 2. Quantitative analysis was done using qRT-PCR, and the results are shown in Fig. 4. Throughout the growth and development of peach fruit, the changing trend of the relative expression of each gene (Fig. 4) was consistent with the changing trend of the gene expression level in the flesh as shown in RNA-seq data, which proves the accuracy of the RNA-seq data. The expression of genes *Prupe.3G100800* (*HCT*) and *Prupe.3G107300* (*4CL*) in the three cultivars showed a high degree of consistency with the total content of CGAs, so they are key genes for the synthesis of CGAs in peach flesh.

3.4 The family similarity and phylogenetic analysis of PpHCT proteins in peach

The results of the phylogenetic analysis showed that the nine HCTs in peach and the reported HCT1 (JQ280303) and HCT3 (JQ280305) in pear aggregated into a large independent group, and the other group was composed of two separate branches of HQT and HCT proteins from other

Table 1 Correlation analysis of total CGA concentration and the expression of CGA metabolism-related genes

Gene ID	Gene Name	Ruiguang 18	Heiyoutao	Beijingyixianhong
Prupe.6G235400	*PpPAL*	− 0.837	0.608	0.443
Prupe.6G040400	*PpC4H*	0.811	− 0.094	0.608
Prupe.1G087900	*Pp4CL*	0.796*	− 0.309	0.862
Prupe.3G107300	*Pp4CL*	0.991**	0.954*	0.731*
Prupe.6G109000	*Pp4CL*	0.348	− 0.715	− 0.522
Prupe.1G580200	*PpC3'H*	− 0.511	− 0.363	0.639
Prupe.1G580500	*PpC3'H*	0.783	0.744*	0.159
Prupe.3G100800	*PpHCT*	0.862*	0.721*	0.731*
Prupe.3G101900	*PpHCT*	0.752	0.796*	0.866
Prupe.3G101600	*PpHCT*	0.862	0.138	0.291
Prupe.3G101700	*PpHCT*	0.114	0.496	0.011
Prupe.3G101800	*PpHCT*	− 0.645	− 0.114	− 0.622

*Indicates significant differences at the 0.05 level

**Indicates greatly significant differences at the 0.01 level

Table 2 Primer sequences in real-time quantitative PCR

Gene ID	Gene Name	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
Prupe.1G087900	*Pp4CL*	ATACTGGTGCTTCGCTTCCC	GTGCAACCAGCGCTCTTTAT
Prupe.3G107300	*Pp4CL*	CATTTGCCAAGCAGCCCTTT	ATGGTTGCCCTTGTGGACTC
Prupe.1G580500	*PpC3'H*	CTCTCCCAAGAGGATCGAGG	GCCTTGTTATGTTGTTGAAAGCC
Prupe.3G100800	*PpHCT*	CACCATCGAGGATTTCGGCG	CTACACGATGTTCCAGGCCA
Prupe.3G101900	*PpHCT*	GCCGTTGATTACTCCGCTGG	GATGAACGGCGGAAGTGTAATG
–	*PpTEF2*	GGTGTGACGATGAAGAGTGATG	TGAAGGAGAGGGAAGGTGAAAG

species (Fig. 5). The key enzyme gene *Prupe.3G100800* (*PpHCT*) screened in this study is highly similar to the two genes *HCT1* (*JQ280303*) and *HCT3* (*JQ280305*) in pear but different from the key enzyme genes HCT and HQT for CGA synthesis in other species.

4 Discussion

Studies have shown that the content of CGAs in fruit was very high in the young fruit stage of apple, decreased rapidly at the middle stage of fruit development, and then decreased more slowly at the late stage (Zhou et al. 2013; Awad and Jager 2001), while in the fruit of pear, CGA content decreased gradually (He et al. 2017).

It is already known that the CGA content in ripe fruit varies significantly in different peach cultivars (Yan et al 2014a). Some reports suggested that the relative content of the two isomers 5-*O*-caffeoylquinic acid and 3-*O*-caffeoylquinic acid may vary in different cultivars. Yan et al. (2014a) first mentioned that the main CGA component in the fruit of white and yellow flesh peach was 5-*O*-caffeoylquinic acid, while 3-*O*-caffeoylquinic acid content in red flesh peach fruit was higher. The same results were obtained in this study. In the ripe fruit of the yellow flesh peach 'Ruiguang 18', the CGAs were significantly lower than those in the red flesh peach 'Heiyoutao' and 'Beijingyixianhong'. The content of 5-*O*-caffeoylquinic acid in 'Ruiguang 18' was slightly higher than that of 3-*O*-caffeoylquinic acid, while in the other two cultivars, the content of the latter was higher than the former. This may be caused by the difference of CGA isomerase activity in peaches with different flesh colors. Further research should be done in the future to determine this.

The accumulation of CGAs during the development of peach fruit varied among cultivars, too. CGA content in most of the tested peach cultivars gradually decreased as the peach fruit developed. A few cultivars, such as 'Redhaven', had a fluctuating content of CGAs, which reached the highest at 94 DAFB, decreased at 100 DAFB, and then increased again at maturity. Some red flesh peach cultivars showed an increase in the early stage and a decline in the middle and late stages (Orazem et al. 2013; Zhang et al. 2018a). Similar results were obtained in this study when testing the CGA content of three peach cultivars. Some reports suggested that the stability of phenols is closely related to pH value. Phenols may degrade due to pH value changes (Zhu et al. 2016). Further research should be done by determining the pH value of fruit flesh from different cultivars while

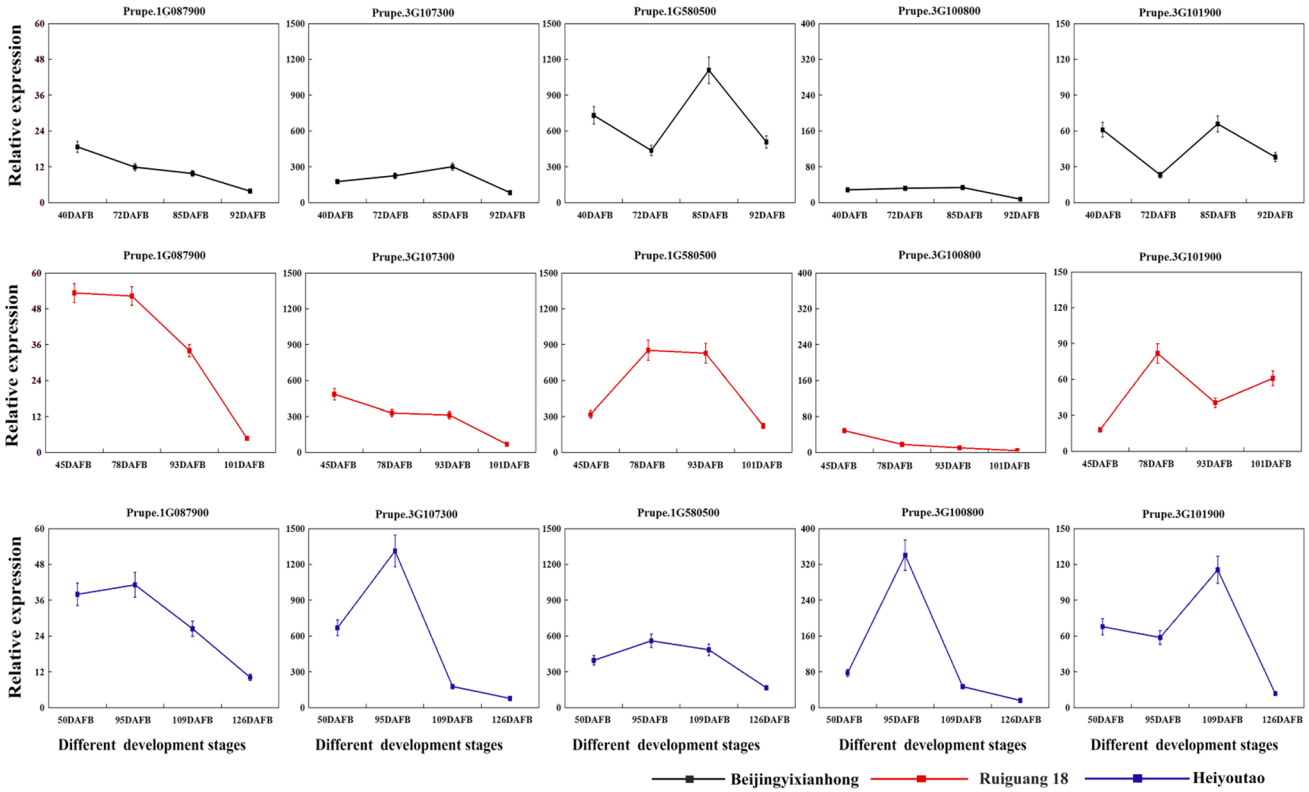

Fig.4 qRT-PCR validation of the candidate genes of CGA synthesis in peach flesh

measuring CGA content and recording the accumulation trend to find out if pH value is one of the key factors.

There are few studies on the molecular mechanism of CGAs metabolism in fruit. The results of the pear study showed that the expression of nine genes, including *PbPAL1*, *PbPAL2*, *PbC3H*, *PbC4H*, *Pb4CL1*, *Pb4CL2*, *Pb4CL6*, *PbHCT1*, and *PbHCT3*, were related to the accumulation and metabolism of CGAs, in which *PbHCT1* (GenBank accession: JQ280303) and *PbHCT3* (GenBank accession: JQ280305) were closely and positively related to the synthesis of CGAs in pear (He et al. 2017). In our study, candidate genes were screened by combining bioinformatics analysis and transcriptome data, and qRT-PCR analysis was performed to verify that the expression patterns of *Prupe.3G100800* (PpHCT) and Prupe3G107300 (4CL) are closely correlated with CGA content in peach, which might play key roles in the synthesis of CGAs in peach flesh.

Both HCT and HQT are key enzymes for the synthesis of CGAs in plants (Mudau et al. 2018). HQT genes positively regulate the synthesis of CGAs in plants such as tobacco, tomato, and honeysuckle (Chen et al. 2017; Niggeweg et al. 2004). HCT and HQT both play important roles in the synthesis of CGAs in artichoke (Sonnante et al. 2010). HCT has different affinities to organic acid substrates in different plants. In *Arabidopsis thaliana*, tobacco, and tomato, HCT has a higher affinity for shikimic acid than quinic acid. Therefore, species that only contain this enzyme have low CGA content (Hoffmann et al. 2004; Sonnante et al. 2010). The sequence of the *CcHCT* gene in artichoke is similar to that of HCT in tobacco, but its enzyme properties are similar to those of HQT in tobacco and tomato; that is, the affinity with quinic acid is higher than shikimic acid (Comino et al. 2007). The *Prupe.3G100800* (*PpHCT*) gene obtained in this study is highly similar to the key CGA metabolism-related genes *HCT1* (*JQ280303*) and *HCT3* (*JQ280305*) in pear. It may be a key gene for CGA synthesis in peach. The specific enzymatic characteristics and mechanisms should be further studied.

The possible pathways for CGA synthesis can be inferred based on the identification of key genes, their enzymatic properties, and the metabolism of intermediate products, substrates, and competitive products. The silencing of the HQT gene in tobacco and tomato results in a 98% reduction in CGA level, but does not affect lignin formation, so in these species at least, the first two of these routes (Fig. 1) is probably responsible for the biosynthesis and accumulation of CGAs (Niggeweg et al. 2004; Comino et al. 2009; Naveed et al. 2018). By contrast, a lowered HCT expression in tobacco and *Medicago sativa* changes lignin amount and composition, thereby implicating the third pathway (Fig. 1)

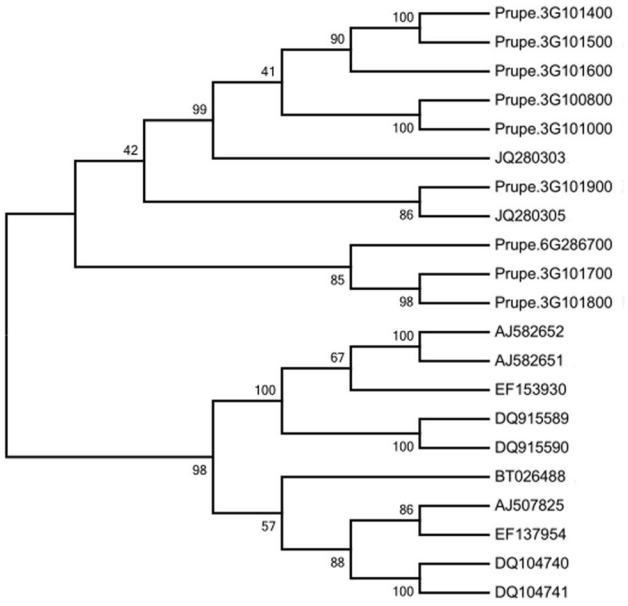

Fig. 5 Phylogenetic analysis of PpHCT proteins from peach and HQT/HCT proteins from other species. The tree was constructed by the neighbour-joining method with 1000 bootstrap replicates. The length of the lines indicates the relative distances between nodes. PpHCT (*Prunus persica*, Prupe.3G100800, Prupe.3G101000, Prupe.3G101400, Prupe.3G101500, Prupe.3G101600, Prupe.3G101700, Prupe.3G101800, Prupe.3G101900, and Prupe.6G286700); PbHCT (*Pyrus bretschneideri*, JQ280303 and JQ280305); AtHCT (*Arabidopsis thaliana*, BT026488); NtHCT (*Nicotiana tabacum*, AJ507825); CcaHCT(*Coffea canephora*, EF137954); CcHCT (*Cynara cardunculus*, DQ104740 and DQ104741); LeHQT (*Lycopersicum esculentum*, AJ582652); NtHQT (*Nicotiana tabacum*, AJ582651); CcaHQT (*Coffea canephora*, EF153930); CcHQT (*Cynara cardunculus*, DQ915589 and DQ915590)

in lignin biosynthesis (Hoffmann et al. 2004; Shadle et al. 2007). No genes with high similarity to the HQT gene were found in peach in this study. And according to previous reports, the main component of organic acids in peach is quinic acid instead of shikimic acid (Zheng et al. 2020). It was deduced that *PpHCT* (Prupe.3G100800), a key enzyme in peach flesh, may be more likely to have an affinity with quinic acid, similar to HQT in tobacco and tomato. At the same time, the synthesis pathway of CGAs in peach may be the second pathway (Fig. 1); that is, p-coumarin-shikimic acid might be synthesized under the action of PpHCT, with p-coumarolyl coenzyme A and quinic acid as the substrates. Then, p-coumarin-shikimic acid might be hydroxylated by PpC3'H to form CGAs. This deduction needs to be verified through research on substrates (such as quinic acid and shikimic acid), accumulation of intermediate products (such as p-coumarinic acid, p-coumaroyl shikimic acid, and caffeoyl shikimic acid) or competing products (such as lignin), and the zymologic functions of the key enzyme *PpHCT* (Prupe.3G100800) during peach fruit development.

5 Conclusion

CGAs not only have a large impact on the taste of peach fruit, which contribute a lot to the overall fruit quality, but also have important physiological functions. The CGA metabolism pathway is unclear in peach, and unraveling the molecular mechanism(s) underlying it could be useful for breeding purposes. This research describes changes in CGA contents in peach fruit during development and their relation with biosynthesis-related gene expression. Changes in CGA content varied in different peach cultivars. *Prupe.3G100800* (*PpHCT*) and *Prupe3G107300* (4CL) were probably the key genes for the synthesis of CGAs in peach flesh. It could be inferred that CGAs might be synthesized in peach fruit flesh via the hydroxylation of p-coumarolylquinic acid.

Acknowledgements This work was supported by funds of Natural Science Foundation of Jiangsu Province (BK20200278); China Agriculture Research System (CARS-30); Species Conservation Project of Ministry of Agriculture and Rural Affair (19190156); National Crop Germplasm Resources Infrastructure in China (NHGRC2020-NH16).

Author contributions JY provided the experimental ideas and designed the research; SG, MZ, BZ, and ZC performed the experiments and the analyzed data. JY and ZS drafted the manuscript. ZS, RM, and MY revised the paper. All authors have read and approved the final manuscript.

Declarations

Conflict of interest The authors have declared that they have no conflict of interest.

References

Abdelghafar A, Burrell R, Reighard G, Gasic K (2018) Antioxidant capacity and bioactive compounds accumulation in peach breeding germplasm. J Am Pomol Soc 72:40–69

Awad MA, Jager PK (2001) Flavonoid and chlorogenic acid changes in skin of 'elstar' and 'jonagold' apples during development and ripening. Sci Hortic 90:69–83

Chen ZX, Liu GH, Liu YQ, Xian ZQ, Tang N (2017) Overexpression of the LmHQT1 gene increases chlorogenic acid production in Lonicera macranthoides Hand-Mazz. Acta Physiol Plant 39:27

Comino C, Hehn A, Moglia A, Menin B, Bourguad F, Lanteri S, Portis E (2009) The isolation and mapping of a novel hydroxycinnamoyltransferase in the globe artichoke chlorogenic acid pathway. BMC Plant Biol 9:30

Comino C, Lanteri S, Portis E, Acquadro A, Romani A, Hehn A, Larbat R, Bourgaud F (2007) Isolation and functional characterization of a cDNA coding a hydroxycinnamoyl transferase involved in phenylpropanoid biosynthesis in Cynara cardunculus L. BMC Plant Biol 7:14

Chang S, Tan C, Frankel NF, Barrett DM (2000) Low-density lipoprotein antioxidant activity of phenolic compounds and polyphenol oxidase activity in selected clingstone peach cultivars. J Agric Food Chem 48:147–151

Cui T, Nakamura K, Ma L, Li JZ, Kayahara H (2005) Analyses of arbutin and chlorogenic acid, the major phenolic constituents in oriental pear. J Agric Food Chem 53:3882–3887

Florea L, Song L, Salzberg SL (2013) Thousands of exon skipping events differentiate among splicing patterns in sixteen human tissues. F1000 Res 2:188

Hammerschmidt R (2014) Chlorogenic acid: a versatile defense compound. Physiol Mol Plant Pathol 88:iii–iv

He JG, Chang YD, Guan JF, Ge WY, Zhao Z (2017) Changes of chlorogenic acid content and its synthesis-associated genes expression in Xuehua pear fruit during development. J Integr Agric 16:471–477

Hoffmann L, Besseau S, Geoffroy P, Ritzenthaler C, Meyer D, Lapierre C, Pollet B, Legrand M (2004) Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyl transferase Affects phenylpropanoid biosynthesis. Plant Cell 16:1446–1465

Jaiswal R, Heiko M, Anja M, Karar MGE, Kuhnert N (2014) Identification and characterization of chlorogenic acids, chlorogenic acid glycosides and flavonoids from Lonicera henryi L. (Caprifoliaceae) leaves by LC-MSn. Phytochemistry 108:252–263

Jiao WX, Li XX, Wang XM, Cao JK, Jiang WB (2018) Chlorogenic acid induces resistance against, Penicillium expansum, in peach fruit by activating the salicylic acid signaling pathway. Food Chem 260:274–282

Jung S, Lee T, Cheng CH, Buble K, Zheng P, Yu J, Humann J, Ficklin SP, Gasic K, Scott K, Frank M, Ru S, Hough H, Evans K, Peace C, Olmstead M, DeVetter LW, McFerson J, Coe M, Wegrzyn JL, Staton ME, Abbott AG, Main D (2019) 15 years of GDR: new data and functionality in the Genome Database for Rosaceae. Nucleic Acids Res 47:D1137–D1145

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408

Lombardo VA, Osorio S, Borsani J, Lauxmann MA, Bustamante CA, Budde CO (2011) Metabolic profiling during peach fruit development and ripening reveals the metabolic networks that underpin each developmental stage. Plant Physiol 157:1696–1710

Lu JF, Liu SY, Lu W, Xi WP (2017) Phenolic profiles and antioxidant activity of fruit pilp from different type of peaches. Sci Agric Sin 50:3205–3214

Martinez G, Regente M, Jacobi S, Rio M, Del Pinedo M, Delacanal L (2017) Chlorogenic acid is a fungicide active against phytopathogenic fungi. Pestic Biochem Physiol 140:30–35

Mudau SP, Steenkamp PA, Piater LA, Palma M, De Tucci M, Madala NE, Dubery IA (2018) Metabolomics-guided investigations of unintended effects of the expression of the hydroxycinnamoyl quinate hydroxycinnamoyl transferase (hqt1) gene from Cynara cardunculus var. scolymus in Nicotiana tabacum cell cultures. Plant Physiol Biochem 127:287–298

Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, Ahmad F, Babazadeh D, Xia FF, Modarresi GF, Li WH, Zhou XH (2018) Chlorogenic acid (CGA): a pharmacological review and call for further research. Biomed Pharmacother 97:67–74

Niggeweg R, Michael AJ, Martin C (2004) Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat Biotechnol 22:746–754

Orazem P, Mikulic-Petkovsek M, Stampar F, Hudina M (2013) Changes during the last ripening stage in pomological and biochemical parameters of the 'Redhaven' peach cultivar grafted on different rootstocks. Sci Hortic 160:326–334

Shadle G, Chen F, Reddy MS, Jackson L, Nakashima J, Dixon RA (2007) Down-regulation of hydroxycinnamoyl CoA: Shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality. Phytochemistry 68:1521–1529

Sonnante G, Damore R, Blanco E, Pierri CL, Palma M, De Luo J, Tucci M, Martin C (2010) Novel hydroxycinnamoyl-coenzyme A quinate transferase genes from artichoke are involved in the synthesis of chlorogenic acid. Plant Physiol 153:1224–1238

Tong ZG, Gao ZH, Wang F, Zhou J, Zhang Z (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 10:1–3

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, Baren MJ, Van Salzberg SL, Wold BJ, Pachter L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28:511–515

Tomas-Barberan FA, Gil MI, Cremin P, Waterhouse AL, Hess-Pierce B, Kader AA (2001) HPLC-DAD-ESIMS Analysis of phenolic compounds in nectarines, peaches, and plums. J Agric Food Chem 49:4748–4760

Villarino M, Sandin-Espana P, Melgarejo P, De Cal A (2011) High chlorogenic and neochlorogenic acid levels in immature peaches reduce Monilinia laxa infection by interfering with fungal melanin biosynthesis. J Agric Food Chem 59:3205–3213

Vitus IO, Juan JB, Josep U, Yolanda G (2019) Breeding strategies for identifying superior peach genotypes resistant to brown rot. Sci Hortic 246:1028–1036

Yan J, Song ZZ, Cai ZX, Shen ZJ, Ma RJ, Yu ML (2018) Proanthocyanidin accumulation in peach fruit with three types of flesh color Jiangsu. Agric J 34:651–656

Yan J, Cai ZX, Shen ZJ, Zhang BB, Qian W, Yu ML (2014a) Determination and comparison of 10 phenolic compounds in peach with three types of flesh color. Acta Horticult Sin 41:319–328

Yan J, Shen ZJ, Cai ZX, Yu ML (2014b) Advances of study on phenolic compounds in peach fruit. J Fruit Sci 31:477–485

Zhang BB, Shen ZJ, Ma RJ, Jiang H, Yan J, Yu ML (2018a) Antioxidant capacity evaluation of peach fruit based on flesh individual phenol and total phenol content. Acta Horticult Sin 45:121–132

Zhang BB, Shen ZJ, Ma RJ, Yan J, Yu ML (2018b) Grey relational analysis and fuzzy synthetic discrimination of antioxidant components in peach fruit. Arch Biol Sci 70:449–458

Zheng BB, Zhao L, Jiang XH, Cherono S, Liu JJ, Ogutu C, Ntini C, Zhang XJ, Han YP (2020) Assessment of organic acid accumulation and its related genes in peach. Food Chem 334:127567

Zhou L, Kang GD, Wang Q, Zhang LY, Zhang CX, Tian Y, Cong PH (2013) Analysis of chlorogenic acid content in Apple Fruit during development. China Fruits 3:36–38

Zhu P, Miao XL, Chen Y (2016) Degradation kinetics of chlorogenic acid, cryptochlorogenic acid, and neochlorogenic acid at neutral and alkaline pH values. Acta Pharm Sin 51:122–126