Whole-transcriptome Analysis Reveals the Promising Bioresource of Total Flavonoid from Bitter-Pit Apples, Especially the Pitted Parts

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Abstract

Background: Apple fruits are rich in flavonoids, and play important roles in human-health protection against chronic diseases. However, bitter pit in apple has affected apple fruit production worldwide. There must be some application values could be exploited from the bitter-pit apples so as to reduce the loss caused by bitter pit.

Results: In the present study, the influence of bitter pit on the total flavonoid content and flavonoid biosynthesis in apples was investigated using the aluminum chloride colorimetric method, whole-transcriptome sequencing and qRT-PCR analysis. The results showed that the total flavonoid content in bitter-pit apples (BG), pitted parts (BGBB) and non-pitted parts (JKBF) was 4.28-fold, 4.68-fold and 0.57-fold respectively as that in healthy apples (JKG). By RNA-Seq analysis, 26, 23 and 3 DEGs involved in flavonoid biosynthesis were enriched in JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF comparisons, respectively. Eight DEGs [*CYP98A3(1)*, *CYP98A3(2)*, *BADH*, *DAT*, *HCT(1)*, *HCT(2)*, *CHI(1)* and *CHI(2)*], were selected to be validated by qRT-PCR analysis, and the consistent expression patterns with RNA-Seq analysis were obtained, the results showed that the 8 DEGs were upregulated in BG and BGBB but downregulated in JKBF when compared with JKG.

Conclusions: The flavonoid accumulation and biosynthesis in apples, especially the pitted parts, were stimulated greatly by bitter pit, while depressed slightly in non-pitted parts. The results indicated that the bitter-pit apples, especially the pitted parts, could be used as the promising bioresource of total flavonoid for the therapeutic utilization in human chronic diseases.

Background

Flavonoids are an important group of polyphenolic compounds that are widely distributed throughout the plant kingdom, especially in colored fruit and flowers [1–3]. Over 10,000 structures of flavonoids have been identified, and these structures are divided into several subclasses, including flavones, flavanes, flavanones, flavonols, flavanonols, flavan-3-ols, anthocyanidins, aurones, bioflavonoids, chalcones, dihydrochalcones and isoflavones [4].

Flavonoids play important roles in multiple plant functions, such as plant growth, fertility, pigmentation, insect attraction, UV-B protection, the production of phytoalexins, allelochemicals, antioxidants, protection against damage by dormancy, ultraviolet light and phytopathogens, and protection against biotic and abiotic stresses [5, 6]. Flavonoids are often useful to humans by offering protection against chronic diseases, including infant leukemia, cerebrovascular disease, cardiovascular disease, certain types of cancer, diabetes, hepatic ailments, rheumatoid arthritis, aging diseases and inflammatory diseases, due to their antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal and antiviral activities [7–14].

The apple industry is the largest fruit industry in the world because of the high nutritional and pharmaceutical values of apple fruit, such as the prevention of cardiovascular and cerebrovascular diseases and the anticancer and antioxidation properties, due to their richness in free polyphenols, especially flavonoids, which are easily absorbed and used by the human body [15–19]. Bitter pit is a physiological disorder affecting apple fruit production worldwide [20]. The symptoms of bitter pit are characterized by small, dark depressions that are most commonly found in the distal portions of fruit and are caused by the breakdown of flesh cells just beneath the peel [21, 22]. This disorder can develop before and after harvest, usually causes significant economic losses, has puzzled researchers for decades and still remains unresolved [20, 23, 24]. There have been some studies on the influence of biotic and abiotic factors, such as cultivars, strains, pollination combinations, temperature, light, high

pressures and phenylpropanoids on the total flavonoid content in apple fruit [18, 25–31]. However, there have been no reports about the influence of bitter pit on flavonoid biosynthesis and accumulation in apple fruit.

Whole-transcriptome sequencing, which employs next-generation sequencing (NGS) technologies to sequence complementary DNAs (cDNAs), is an advanced and widely-used method for the massively parallel sequencing of total RNA in plants with a higher resolution than Sanger sequencing and microarray-based methods [32–35]. In the present study, the total flavonoid content was determined using the aluminum chloride colorimetric method, then whole-transcriptome sequencing was used to evaluate the differences in total RNA, including messenger RNAs (mRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs) and microRNAs (miRNAs) [36–39], especially genes related to flavonoid biosynthesis, in healthy apple fruit, bitter-pit fruit and different parts of bitter-pit fruit. Finally, the expression of the candidate differentially expressed genes (DEGs) involved in flavonoid biosynthesis was validated by qRT-PCR analysis. The results of this study will provide new insights into the influence of bitter pit on the flavonoid biosynthesis and accumulation in apples by investigating the influence of bitter pit on flavonoid biosynthesis and accumulation in apple fruit and evaluating the potential bioresources of flavonoids for therapeutic utilization from bitter-pit apple fruit.

Results

Total flavonoid content

The total flavonoid content in apple fruit was determined using the aluminum chloride colorimetric method, and the results showed that the total flavonoid content in BG and BGBB was significantly higher than that in JKG, with ratios of 4.28 (BG/JKG) and 4.68 (BGBB/JKG), respectively, while the total flavonoid content in JKBF was lower than that in JKG, with a ratio of 0.57 (JKBF/JKG) (Fig. 1). At the same time, we found the total flavonoid content in BGBB was a little higher than that in BG, with ratio of 1.09 (BGBB/BG), and the total flavonoid content in JKBF was significantly lower than that in BG and BGBB, with ratios of 7.55 (BG/JKBF) and 8.26 (BGBB/JKBF), respectively.

RNA-Seq and quality filtering

Healthy and bitter-pit apple fruit of uniform ripeness and size were chosen to study the influence of bitter pit on different expression of flavonoid biosynthesis-related genes. Twelve cDNA libraries of the whole transcriptome were generated for RNA-Seq. We obtained 963.948 M raw reads containing 144.592 G nucleotides by RNA-Seq. After clean-up and quality filtering, 862.772 M clean reads containing 124.435 G nucleotides were obtained (Table 1). The clean Q30 percentages were 94.1%, indicating that the RNA-seq results were of high quality and suitable for use in further analyses. By miRNA sequencing, 4331 reads associated with 3753 target genes were obtained, among which 2571 genes were upregulated and 1182 genes were downregulated. Of the 41894 total RNA sequences subjected to sequence analysis and annotation, 15852 were known, and 26042 were novel (Table 2).

Table 1
The quality control of whole-transcriptome RNA-Seq stats

Quantity (Percentage)	Raw Reads (M)	Raw Bases (G)	Raw Q20 (G)	Raw Q30 (G)	Clean Reads (M)	Clean Bases (G)	Clean Q20 (G)	Clean Q30 (G)	Average Length (bp)
JKG_1	93.383	14.007	13.308 (95.0%)	12.528 (89.4%)	82.488 (88.3%)	11.988 (85.6%)	11.787 (98.3%)	11.388 (95.0%)	145.3
JKG_2	75.388	11.308	10.721 (94.8%)	10.095 (89.3%)	66.332 (88.0%)	9.559 (84.5%)	9.412 (98.5%)	9.108 (95.3%)	144.1
JKG_3	88.231	13.235	12.573 (95.0%)	11.856 (89.6%)	77.882 (88.3%)	11.260 (85.1%)	11.084 (98.4%)	10.730 (95.3%)	144.6
BG_1	81.606	12.241	11.703 (95.6%)	11.063 (90.4%)	73.073 (89.5%)	10.652 (87.0%)	10.481 (98.4%)	10.140 (95.2%)	145.8
BG_2	63.588	9.538	9.133 (95.8%)	8.649 (90.7%)	57.173 (89.9%)	8.378 (87.8%)	8.250 (98.5%)	7.991 (95.4%)	146.5
BG_3	80.519	12.078	11.575 (95.8%)	10.966 (90.8%)	72.705 (90.3%)	10.596 (87.7%)	10.433 (98.5%)	10.103 (95.3%)	145.7
BGBB_1	77.476	11.621	11.026 (94.9%)	10.333 (88.9%)	70.019 (90.4%)	10.038 (86.4%)	9.857 (98.2%)	9.485 (94.5%)	143.4
BGBB_2	78.607	11.791	11.187 (94.9%)	10.464 (88.7%)	69.857 (88.9%)	10.070 (85.4%)	9.871 (98.0%)	9.485 (94.2%)	144.1
BGBB_3	88.091	13.214	12.487 (94.5%)	11.638 (88.1%)	79.116 (89.8%)	11.347 (85.9%)	11.130 (98.1%)	10.681 (94.1%)	143.4
JKBF_1	76.206	11.431	10.844 (94.9%)	10.159 (88.9%)	68.899 (90.4%)	9.927 (86.8%)	9.747 (98.2%)	9.378 (94.5%)	144.1
JKBF_2	84.28	12.642	12.013 (95.0%)	11.258 (89.0%)	75.281 (89.3%)	10.809 (85.5%)	10.603 (98.1%)	10.199 (94.4%)	143.6
JKBF_3	76.573	11.486	10.984 (95.6%)	10.359 (90.2%)	69.947 (91.3%)	9.811 (85.4%)	9.638 (98.2%)	9.297 (94.8%)	140.3

Table 2
Transcript types of RNA-Seq stats

Class	mRNA	lncRNA	circRNA	miRNA	Others	Unknown	Total
Known	12645	0	52	3017	0	138	15852
Novel	20937	1860	46	2877	322	0	26042
Total	33582	1860	98	5894	322	138	41894

The RNA-Seq data were submitted to SRA database in NCBI with the accession number of PRJNA640254.

Analysis of sequence length distribution

The sequence length distribution of the transcripts is depicted by FPKM values in Table 3. FPKM was used to quantify gene expression, as this method eliminates the effect of different gene lengths and sequencing levels on the gene expression calculation. For the fruit samples of JKG, BG, BGBB and JKBF, there were 17086, 16396, 16736 and 17401 transcripts with an FPKM value < 5.0, accounting for 48.17%, 45.80%, 46.51% and 49.17% of the total, respectively. There were 3874, 4044, 4178 and 3688 transcripts with FPKM values > 50.0, accounting for 10.92%, 11.3%, 11.61% and 10.42% of the total, respectively.

Table 3
Sequence length distribution of transcripts and genes assembled from Illumina reads

FPKM Value (Percentage)		0-0.5	0.5-1.0	1.0-5.0	5.0-10.0	10.0-50.0	> 50.0	Total
Transcripts	JKG	3402 (9.59%)	2866 (8.08%)	10818 (30.50%)	5356 (15.10%)	9156 (25.81%)	3874 (10.92%)	35472
	BG	2354 (6.58%)	2801 (7.82%)	11241 (31.40%)	5664 (15.82%)	9694 (27.08%)	4044 (11.30%)	35798
	BGBB	1363 (3.79%)	3090 (8.59%)	12283 (34.14%)	5563 (15.46%)	9506 (26.42%)	4178 (11.61%)	35983
	JKBF	3493 (9.87%)	2868 (8.1%)	11040 (31.2%)	5304 (14.99%)	8996 (25.42%)	3688 (10.42%)	35389
Genes	JKG	1580 (6.00%)	1348 (5.12%)	6454 (24.52%)	4282 (16.27%)	8694 (33.03%)	3963 (15.06%)	26321
	BG	816 (3.07%)	1208 (4.55%)	6646 (25.01%)	4554 (17.14%)	9254 (34.82%)	4099 (15.42%)	26577
	BGBB	337 (1.27%)	1214 (4.56%)	7196 (27.03%)	4519 (16.97%)	9141 (34.33%)	4219 (15.85%)	26626
	JKBF	1699 (6.46%)	1356 (5.16%)	6536 (24.86%)	4326 (16.46%)	8604 (32.73%)	3768 (14.33%)	26289

Similar results were obtained for the sequence-length distribution of genes (Table 3). For the fruit samples of JKG, BG, BGBB and JKBF, there were 9382, 8670, 8747 and 9591 genes with FPKM values < 5.0, accounting for 35.64%, 32.62%, 32.85% and 36.48% of the total, respectively. There were 3963, 4099, 4219 and 3768 genes with FPKM values > 50.0, accounting for 15.06%, 15.42%, 15.85% and 14.33% of the total, respectively.

Analysis of DETs and DEGs

The expression patterns of transcripts in healthy and bitter-pit apple fruits were investigated using pairwise comparisons. By whole-transcriptome sequencing, 35920, 35990 and 35714 transcripts were obtained from JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF, respectively, among which 2577, 4081 and 776 transcripts, respectively, were differentially expressed. Compared with JKG, BG, BGBB and JKBF had 2085, 2774 and 325 upregulated differentially expressed transcripts (DETs), respectively, and 492, 1307 and 154 downregulated DETs, respectively (Table 4).

Table 4
The DETs and DEGs obtained from JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF

Treatment	DETs			DEGs			miRNA		
	Total	Up	Down	Total	Up	Down	Total	Up	Down
JKG vs. BG	2577	2085	492	2498	2035	463	134	45	89
JKG vs. BGBB	4081	2774	1307	3847	2680	1167	157	56	101
JKG vs. JKBF	776	325	451	774	337	437	137	34	103

Correspondingly, 26600 shared genes were obtained from JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF, among which 2498, 3847 and 774 genes, respectively, were differentially expressed (Table 4). Compared with JKG, BG, BGBB and JKBF had 2035, 2680 and 337 upregulated DEGs, respectively, and 463, 1167 and 437 downregulated DEGs, respectively. For miRNA sequencing, 134, 157 and 137 miRNAs DEGs were obtained from JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF, respectively (Table 4). Compared with JKG, BG, BGBB and JKBF had 45, 56 and 34 upregulated DEGs, respectively, and 89, 101 and 103 downregulated DEGs, respectively.

In summary, a total of 2632, 4004 and 911 DEGs were obtained from JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF, respectively, of which 2080, 2736 and 371 DEGs were upregulated and 552, 1268 and 540 DEGs were downregulated.

GO enrichment analysis of DEGs

For the JKG vs. BG comparison, a total of 4643 genes (3922 upregulated and 721 downregulated) were highly enriched in 3 classes and 34 sub-classes, of which 1065, 2632 and 946 genes were annotated with GO terms related to “molecular function”, “biological process” and “cellular component”, respectively (Fig. 2a). For the JKG vs. BGBB comparison, a total of 4760 genes (3121 upregulated and 1639 downregulated) were highly enriched in 3 classes and 32 sub-classes, of which 1357, 2605 and 798 genes were annotated with GO terms related to “molecular function”, “biological process” and “cellular component”, respectively (Fig. 2b). For the JKG vs. BG comparison, a total of 2156 genes (501 upregulated and 1655 downregulated) were highly enriched in 3 classes and 34 sub-classes, of which 457, 1107 and 592 genes were annotated with GO terms related to “molecular function”, “biological process” and “cellular component”, respectively (Fig. 2c).

KEGG enrichment analysis of DEGs

In the KEGG pathway enrichment analysis, 60 pathways corresponding to 646 DEGs, 73 pathways corresponding to 788 DEGs and 31 pathways corresponding to 109 DEGs were significantly enriched in the JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF comparisons, respectively, of which 26 DEGs (0 downregulated and 24 upregulated), 23 DEGs (0 downregulated and 23 upregulated) and 3 DEGs (3 downregulated and 0 upregulated) involved in flavonoid biosynthesis were enriched (Fig. 3).

The flavonoid biosynthesis pathway in KEGG database from JKG vs. BG (Fig. 4a), JKG vs. BGBB (Fig. 4b) and JKG vs. JKBF (Fig. 4c) indicated that the DEGs involved in flavonoid biosynthesis were upregulated in JKG vs. BG and JKG vs. BGBB while downregulated in JKG vs. JKBF, which was consistent with the KEGG pathway enrichment analysis.

qRT-PCR Validation and expression analysis of DEGs

To confirm the reproducibility and accuracy of the differential gene expression identified through Illumina analysis, 8 DEGs [*CYP98A2* (1), *CYP98A2* (2), *BADH*, *DAT*, *MdHCT* (1), *MdHCT* (2), *CHI* (1) and *CHI* (2)] related to flavonoid biosynthesis [ko00941] with fold-changes ≥ 1 were selected for qRT-PCR validation (Table 5). The expression patterns of these genes according to RNA-Seq and qRT-PCR are shown in Table 6.

Table 5
The qRT-PCR primers for the 8 selected genes

Gene ID	Gene Name	Predicted Function	Primers (5'-3')
MD08G1242900	<i>CYP98A2</i> (1)	cytochrome P450 98A2-like (LOC103441965)	F: AACCACTGCACCAAACCTGA R: AGCGATCCGCCTATCTTCAC
MD15G1436500	<i>CYP98A2</i> (2)	cytochrome P450 98A2-like (LOC103441965)	F: CGGTATCAACTTGGTGGCCT R: CGTCCCTGGATTTTCGGACA
MD05G1219000	<i>BAHD</i>	BAHD acyltransferase At5g47980-like	F: GCGGATGTTTCCCACTCA R: CAGCCCCAAATGCTACGAGA
MD16G1110600	<i>DAT</i>	deacetylvindoline O-acetyltransferase-like	F: CAGTGAACCTGCGCACTAGA R: GCAACTCAATCACGCTGTCC
MD17G1224900	<i>MdHCT</i> (1)	Hydroxycinnamoyl-CoA Shikimate O-hydroxycinnamoyl transferase-like	F: AATAAGACCGCACAGACGGA R: GTCGAAGAAGGAGTTGCCCT
MD17G1225100	<i>MdHCT</i> (2)	Hydroxycinnamoyl-CoA shikimate O-hydroxycinnamoyl transferase-like	F: GGTGATTCCGAGCATCCACA R: GCCGCAGTCAATCTCGATAC
MD01G1118000	<i>CHI</i> (1)	chalcone–flavonone isomerase	F: CAGAGAAGCGGGTCTCTCCC R: GCGTTGGAGCCATTTGACAAT
MD01G1118100	<i>CHI</i> (2)	chalcone–flavonone isomerase	F: ATTTCCCACCCGGTGCTT R: CCGGCTTCAGCACCATTAGA
	<i>MdActin</i>	endogenous control	F: TGACCGAATGAGCAAGGAAATTACT R: CTCAGCTTTGGCAATCCACATC

Table 6
RNA-Seq FPKM values, qRT-PCR relative expression levels and ratios of 8 candidate DEGs

Genes Values		<i>CYP98A2</i> (1)	<i>CYP98A2</i> (2)	<i>BADH</i>	<i>DAT</i>	<i>MdHCT</i> (1)	<i>MdHCT</i> (2)	<i>CHI</i> (1)	<i>CHI</i> (2)
RNA- seq	JKB	61.80	2.32	0.37	5.67	60.45	4.04	10.29	0.71
	BG	88.61	25.19	4.25	9.66	405.70	9.13	69.70	20.83
	BGBB	112.91	42.38	6.81	18.12	780.75	16.20	104.78	36.87
	JKBF	27.55	1.62	0.30	1.94	31.10	1.82	7.95	0.46
	BG/JKG	1.43	10.86	11.48	1.70	6.71	2.26	6.77	29.42
	BGBB/JKG	1.83	18.27	18.42	3.20	12.92	4.01	10.18	52.08
	JKBF/JKG	0.45	0.70	0.82	0.34	0.51	0.45	0.77	0.65
qRT- PCR	JKB	1.58	1.71	5.33	1.27	5.29	2.08	4.76	1.83
	BG	1.80	6.41	35.60	7.61	17.97	2.18	17.26	13.14
	BGBB	1.98	6.90	38.04	8.65	24.92	2.23	18.29	14.71
	JKBF	0.65	0.20	5.13	0.85	4.81	0.49	1.83	1.37
	BG/JKG	1.14	3.74	6.69	6.01	3.40	1.05	3.62	7.17
	BGBB/JKG	1.25	4.03	7.14	6.83	4.71	1.07	3.84	8.03
	JKBF/JKG	0.41	0.12	0.96	0.67	0.91	0.24	0.38	0.74

The expression patterns of the 8 candidate genes were determined by RNA-Seq and analyzed to determine the FPKM values. The results showed that these genes were upregulated in JKG vs. BG and JKG vs. BGBB, with FPKM ratios of 1.43–29.42 and 1.83–52.08, respectively, while they were downregulated in JKG vs. JKBF, with FPKM ratios of 0.34–0.82. The expression levels of the 8 candidate genes were validated by qRT-PCR analysis, and the results showed that the relative expression levels of these genes were increased in JKG vs. BG and JKG vs. BGBB comparisons, with ratios of 1.05–7.17 and 1.07–8.03, respectively, while the relative expression levels were decreased in JKG vs. JKBF comparison, with expression ratios of 0.12–0.96.

Discussion

Flavonoids constitute a large group of secondary metabolites that are widely distributed in plants, they play important roles in many biological processes, and possess several health-promoting and health-protecting properties due to their antioxidant, anticancer, antifungal and antiviral activities [1–14].

Apples have been reported to be rich in flavonoids, and the biosynthesis and accumulation of the total flavonoid content in apple fruit are influenced by many biotic and abiotic factors [16, 19, 25–31]. Abid *et al.* [26] observed a significant increase in the total amount of phenolic compounds and flavonoids of apple juice after high-pressure processing (HPP) at 450 MPa/25 °C/10 min. Fernández-Jalao *et al.* [31] observed that high-pressure processing (400 MPa/35 °C/5 min) increased 30% of total flavonols and maintained total phenolic compounds in S-apples, and increase 54% of total phenolic compounds in I-apple treated at 600 MPa/35 °C/5 min. Lower temperatures and

increased exposure to light during maturity and harvest might improve the total phenolic and total flavonoid content in the apple peel [25, 28]. Liu *et al.* [19] found that the total flavonoid content in the peels and pulps of three Xinjiang red-flesh apple lines were 1.23–1.61 times and 1.43–3.49 times higher than those of the control. Shafiq and Singh [29] found that a single preharvest spray application of L-phenylalanine (100 mg L^{-1}), when applied 3–4 weeks prior to the anticipated commercial fruit maturity, increased the accumulation of flavonoids in the fruit skin without adversely affecting the fruit quality. Zhang *et al.* [30] found that, during the whole growth period of fruit development, the flavonoid content in ‘Fuji’ and ‘Starkrimson’ apples treated by differently pollinated trees was 4.24%–19.63% higher than that in control apples, with significant differences among different cultivars.

In the present study, the total flavonoid content was determined using the aluminum chloride colorimetric method, with 4.28-fold, 4.68-fold and 0.57-fold of total flavonoid content in BG, BGBB and JKBF, respectively, than in JKG, indicating that bitter pit significantly stimulates flavonoid biosynthesis and accumulation in bitter-pit apples, especially the pitted parts. Liu *et al.* [19] found that the total flavonoid content in apple peels was 2.14–4.64-fold as that in apple pulp. In this present study, the total flavonoid content in BGBB was a little higher than that in BG, and the total flavonoid content in JKBF was significantly lower than that in BG and BGBB, the results were consistent with that of Liu *et al.* [19], which may due to the location of the pitted part (BGBB) just beneath the peels and the location of the non-pitted part (JKBF) mostly far from the peels.

Many genes are involved in the flavonoid biosynthesis pathway and result in differences in flavonoid content in fruit. Xu *et al.* [27] found significant differences in the flavonoid components and contents among 3 strains, i.e., Hongcui NO.1, Hongcui NO.2 and Hongcui NO.4, which might result from variations in the expression of transcription factors and the structure of genes related to flavonoid biosynthesis. Cao *et al.* [40] found that *PpMYB15* and *PpMYBF1* were involved in regulating flavonol biosynthesis in peach fruit. In the present study, based on the results of whole-transcriptome sequencing, GO enrichment analysis and KEGG pathway enrichment analysis, 8 DEGs related to flavonoid biosynthesis, namely, *CYP98A2* (1), *CYP98A2* (2), *BADH*, *DAT*, *MdHCT* (1), *MdHCT* (2), *CHI* (1) and *CHI* (2), were selected for validation of their differential expression in JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF by qRT-PCR analysis.

In terms of biotechnology, cytochrome P450 (CYP) enzymes show promise in the synthesis of high-value chemicals and natural products [41]. The major function of CYPs is to catalyze the biosynthesis of endogenous compounds such as hormones and flavonoids, and participate in the oxidative metabolism of many exogenous compounds [42]. Plant BAHD acyltransferases constitute a large family of acyl CoA-utilizing enzymes whose products include small volatile esters, modified anthocyanins, constitutive defense compounds and phytoalexins [43]. Deacetylindoline 4-*O*-acetyltransferase (DAT) is the terminal enzyme in the synthesis of the alkaloid vindoline [44]. Hydroxycinnamoyl-coenzyme A (CoA) hydroxycinnamoyl transferases (HCTs) belong to the BAHD acyltransferase family and play important roles in the metabolism of biosynthetic intermediates and some specialized metabolites [45]. Chalcone isomerase (CHI) is an important enzyme in the plant flavonoid biosynthetic pathway that catalyzes bicyclic chalcone into tricyclic (S)-flavanones and ensures adequate substrates for the pathway [46].

In the present study, RNA-Seq analysis and qRT-PCR validation showed that the expression of the 8 selected DEGs in JKG vs. BG, JKG vs. BGBB and JKG vs. JKBF had consistent variation trends, that is, the 8 DEGs involved in flavonoid biosynthesis were upregulated in BG and BGBB but downregulated in JKBF compared with JKG, which were consistent with the result of total flavonoid content determined using the aluminum chloride colorimetric method, indicating that the biosynthesis and accumulation of flavonoids in apple fruit, especially the pitted parts (BGBB), was stimulated by bitter pit disorder.

Conclusions

In summary, the accumulation of the total flavonoid content and the expression of genes involved in flavonoid biosynthesis in bitter-pit apples (BG), especially the pitted parts (BGBB), were significantly stimulated but that in non-pitted parts (JKBF) were slightly decreased by bitter pit when compared to the healthy apples (JKG), indicating that bitter-pit apples, especially the pitted parts, could be used as an alternative promising bioresource of total flavonoids, which are widely used for therapeutic applications in human chronic diseases.

Methods

Apple fruit samples

Apple fruits (*Malus domestica* Borkh. cv. Yanfu2, a bud mutation of 'Fuji' apple by Yantai Fruit Tree Institute of Shandong) were harvested at the commercial maturity stage from an orchard located in Yedian, Mengyin, Shandong Province, China (), and healthy fruit and bitter-pit fruit of uniform ripeness and size were selected for experiments. Twenty grams of apple longitudinal slices except the core were sampled from healthy apples (JKG) and bitter-pit apples (BG) respectively, and 20 g of the pitted parts (BGBB, just include the pitted spot, as little as possible the part without pitted symptom) and non-pitted parts (JKBF, the part at least 1.5 cm away from the pitted spot) were collected from the bitter-pit apples respectively, each sample was repeated for three times. The samples were packed with tinfoil and preserved in liquid nitrogen as soon as possible after collection.

Detection of total flavonoid content

Apple fruit samples weighing 0.5 g were used to detect the total flavonoid content using the aluminum chloride colorimetric method [47] at 415 nm ($\lambda_{\max}$ of quercetin) with a U-3900 UV/VIS spectrophotometer (Hitachi High-Tech Science, Japan). The total flavonoid content from apple fruit samples (fresh weight, FW) was expressed as mg kg^{-1} .

Transcriptomic profiling

The total RNA extraction, preparation of whole-transcriptome libraries and Illumina deep sequencing using the Illumina HiSeq™ 2500 system were performed by Beijing Ori-Gene Science and Technology Corp., LTD (Beijing, PR China).

Total RNA was extracted separately from the 12 samples using TRIzol Reagent (Tiangen Biotech CO., LTD, Beijing, China). RNA purity were assessed by A260/A280 (> 1.8) and A260/A230 (> 1.6) using Nanodrop, and the yield and quality were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and an RNA 6000 Nano LabChip Kit (Agilent, CA, USA), RIN > 7.0. Strand-specific adapters were added to the fragmented RNA (approximately 200 nt in length) before reverse transcription followed the manufacturer's instructions.

Whole-transcriptome libraries (including mRNAs, lncRNAs and circRNAs) were constructed using the Ribo-Zero® rRNA Removal Kit (Plant) (Illumina, San Diego, CA, USA) and NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's instructions. miRNA libraries were constructed according to the protocol of the NEBNext® Small RNA Library Prep Set (New England Biolabs) for Illumina. Libraries were controlled for quality and quantified using the BioAnalyzer 2100 system and qPCR (Kapa Biosystems, Woburn, MA, USA). The resulting libraries were sequenced initially on a HiSeq™ 2500 instrument that generated paired-end reads

of 100/150 nucleotides for mRNAs, lncRNAs and circRNAs and 50 bp for miRNAs. Criteria of $|\log_2(\text{fold-change})| \geq 1$ and $P\text{-value} \leq 0.05$ between the two conditions were used to identify differentially expressed genes (DEGs).

DEGs analysis and KEGG functional enrichment

FPKM, which is able to eliminate the effect of different gene lengths and sequencing levels on the calculation of gene expression [48], was used to quantify gene expression with edgeR software (<http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>). The definitions and descriptions of DEGs were acquired by performing Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using the Goseq R package and KOBAS software, respectively [49, 50]. All DEGs were characterized by GO classification and KEGG pathway enrichment analysis using a hypergeometric test and Bonferroni's correction, with corrected P-values < 0.05 [51]. The GO terms and KEGG pathways of differentially expressed miRNA targets were annotated using the Annot8r annotation tool against the UniProt database [52].

qRT-PCR-based validation of flavonoid biosynthesis-related DEGs

qRT-PCR was conducted to verify the expression of 8 DEGs involved in the flavonoid biosynthesis pathway and to assay the same samples to validate the bioinformatic and RNA-seq results. PCR primers were designed with Primer Premier 6.0 (PREMIER Biosoft International, Palo Alto, CA, USA) based on the nucleotide sequences obtained by transcriptional sequencing. The 8 selected genes and their specific primers are listed in Table 5. qRT-PCR using the previously obtained total RNAs as the templates was performed using the FastQuant RT Kit (with gDNAase) (TIANGEN Biotech CO., LTD, Beijing, China) and $2 \times$ RealStar Green Mixture (GenStar Technologies Company Inc., Chino, CA, USA) in an ABI Prism 7500 Sequence Detector (Applied Biosystems, Waltham, USA). *MdActin* was used as an endogenous control. The $2^{-\Delta\Delta C_t}$ comparative threshold cycle (C_t) method was used to evaluate the relative expression levels of target genes [53]. The values reported represent the averages of 3 biological replicates.

Abbreviations

JKG, the healthy apples; BG, bitter-pit apples; BGBB, the pitted parts of bitter-pit apples; JKBF, non-pitted parts of bitter-pit apples; mRNAs, messenger RNAs; lncRNAs, long noncoding RNAs; circRNAs, circular RNAs; miRNAs, microRNAs; FPKM, fragments per kilobase of exon per million mapped reads; DEGs, differentially expressed genes; DETs, differentially expressed transcripts; GDR, Genome Database for Rosaceae; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; FW, fresh weight.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

The RNA-Seq datasets used in the current study are available on the NCBI Short Read Archive Project - PRJNA640254 (<http://www.ncbi.nlm.nih.gov/bioproject/PRJNA640254>).

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

This study was performed by the help of all authors. XM Yu and JZ Wang conceived and designed the experiments; XM Yu performed the experiment, analyzed the data and drafted the manuscript with the help of JZ Wang, XM Xue, PX Nie, R Chen and XP Han. All authors read and approved the final manuscript.

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Figures

Figure 1

Total flavonoid content of different apple fruit samples

Figure 2

GO enrichment analysis of DEGs from JKG vs. BG (2a), JKG vs. BGBB (2b) and JKG vs. JKBF (2c)

Figure 3

KEGG enrichment analysis of DEGs from JKG vs. BG, JKG vs. BG and JKG vs. JKBF

Figure 4

Flavonoid biosynthesis pathway in KEGG from JKG vs. BG (4a), JKG vs. BGBB (4b) and JKG vs. JKBF (4c). (Upregulated Downregulated)