Journal of King Saud University – Science 33 (2021) 101605

Contents lists available at ScienceDirect

Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

Original article

# Transcriptomic and metabolomics of flavonoid compounds in *Actinidia arguta* var. *arguta*

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ARTICLE INFO

*Article history:*
Received 25 February 2021
Revised 6 June 2021
Accepted 7 September 2021
Available online 17 September 2021

*Keywords:*
*Actinidia arguta* var. *arguta*
Flavonoids biosynthesis
Gene expression
Transcriptomics

ABSTRACT

*Actinidia arguta* (Sieb. et Zucc.) Planch ex Miq. is fruit plant with high added value, rich in bioactive compounds relevant for human health, like flavonoids and vitamin C, and has high antioxidant activity. These flavonoids also play important roles in plant immune responses and have strong medicinal implications. Moreover, it is very widely distributed in China and attracted more attention with great economic significance in recent years. However, the majority of research on flavonoids has been focused on fruit, not much has been elucidated in leaf in *A. arguta*. Whether are the leaves of *Actinidia arguta* also rich in flavonoids and other biologically active ingredients? The metabolome and transcriptome in fruits and leaves of *A. arguta* var. *arguta* cv. Changjiang No.1 (CJ-1) was analyzed in this study. We detected ten flavonoids in CJ-1 leaves that belonged to quercetin, isorhamnetin and kaempferol and using LC-MS/MS technology. Flavonoid quercetin were all detected in five tissues. Flavonoid qualification indicated that total flavonoids in young apical leaves were the highest (10219.84 $\mu g \cdot g^{-1}$) and 129.8 folds than in fruits (78.75 $\mu g \cdot g^{-1}$). From the transcriptome data, we obtained 70,631 non-redundant unigenes in total. There were 32,593 differentially expressed genes (DEGs) to be identified, including 29,617 up-regulated and 2976 down-regulated genes in fruits and young apical leaves. There were 127 metabolic pathways enriched with 3567 DEGs, in which 45 DEGs were associated with flavonoid biosynthesis. Next, the expression of qRT-PCR of key flavonoid pathway genes in leaves were higher than that in other tissues, which was consistent with the higher total flavonoid level. The data provided here were valuable for knowing the molecular mechanisms of flavonoids accumulating in different tissues. Our results were of significant contribution to the literature as they proposed possible new insights of applying leaves in *A. arguta* in the food and healthcare industries for their high content of flavonoids.


## 1. Introduction

Kiwifruit fruits contain relatively high vitamin C, flavonoids, carotenoids, phenolics, various minerals, and other biologically active substances beneficial to human health and the human diet (Ferguson and Huang, 2007; Richardson et al., 2018). In recent years, the cultivation of *A. arguta* has gradually increased in China and its research and utilization in biology and agriculture are also rapidly developing. At present, *A. arguta* is commercially cultivated Korea and eastern Russia as well as some cold areas, but it has been also received a lot of attention in many other regions (Almeida et al., 2018). In consequence, knowledge about its nutrients and relevant genes is of great need.

Flavonoids belonged to a class of secondary metabolites with great importance. They have different roles in plants, such as antioxidants, antibiotics, and UV protectants (Schenke et al., 2019) and 'phytonutrients' (Nile et al., 2017). A large number of

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jksus.2021.101605
1018-3647/

studies have been conducted on the biosynthesis, identification and gene expression of flavonoids in different plants including *Oryza sativa* L. (Kim et al., 2018), apple (Hu et al., 2016), radish (M'mbone et al., 2018). Li et al. (2017) showed that *AcMYB75* was regulated the promoter of ANS (Anthocyanidin Synthase) and involved in red kiwifruit anthocyanin biosynthesis. Extensive research has been conducted in recent years to evaluate the nutritional content and composition of kiwifruit fruits including the flavonoids (Yu et al., 2020; Ozen et al., 2019), anthocyanin (Wang et al., 2019), and vitamin C (Pérez-Burillo et al., 2018). Montefiori et al. (2009) first reported that delphinidin was found in two taxa of *Actinidia* species and also found in *A. chinensis* (Liu et al., 2017). Saeed et al. (2019) confirmed that rutin and catechin were in *A. deleciosia* fruits by HPLC analysis and estimated the total flavonoid content of red kiwifruit (RKF) was richest. However, *A. arguta* has high commercial value for abundant biologically active compounds. The knowledge about its gene sequence information and genome is limited, so knowing more about its genetic knowledge can improve its research depth in the field of production and health.

Up to date, many studies have focused on volatile compounds, pigment genes, pancreatic lipase inhibitor and flavonols in *A. arguta* fruits (Wojdyło and Nowicka, 2019), flowers (Matich et al., 2003), roots (Jang et al., 2009). Li et al. (2018) performed RNA-seqs from fruits of two *A. arguta* cultivars and concluded that LDOX (leucoanthocyanidin dioxygenase) might be a key gene regulating the synthesis of anthocyanins in the pulp of 'Tianyuanhong' (a kind of all-red fruit cultivar) kiwifruit. Untill now, the studies on *A. arguta* mostly focused on the fruit, nor the leaves. Therefore, the objectives of this study were focused on (i) data analysis of flavonoids in *A. arguta* leaves, (ii) exploring the genes related to the metabolic pathway of flavonoids in leaves and fruits of *A. arguta* by RNA-Seq, and (iii) analyzing the expression and regulation patterns of these genes in different tissues by qRT-PCR.

## 2. Materials and methods

### 2.1. Plant materials and sampling

The leaves and fruits of CJ-1 (female plant) was collected from the Germplasm Resource Nursery of Wild Kiwifruit in Shenyang Agricultural University. The samples included fruits (F), young apical leaves (YAL), mature leaves (ML) and petioles (P), as well as other two kinds of leaves, those of expanding to 1/4 to 1/3 (QL) and 1/2 to 2/3 (HL) of the full leaf size. Store the sample in the refrigerator at −80 ℃ after collection. The YAL and F were used for transcriptome sequencing, and the F was as the control (Fig. 1).

### 2.2. Flavonoid determination in CJ-1

According to Gutierrez et al. (2017), the flavonoids from the leaves and fruits of *A. arguta* were extracted and slightly changed. In liquid nitrogen, quickly grind 1 g of fresh sample add 5 mL of 0.2% HCl (v/v) methanol solution, and then place it in a refrigerator at −20 ℃ for 24 h, shaking it every 12 h. The sample was centrifuged for 5 min at 3500 rpm. The supernatant was stored at −20 ℃ after filtering. We performed chromatographic analysis by an Agilent 6410 Triple Quad LC-MS/MS (Agilent Technologies, United States), which was equipped with electron spray ionization (ESI) and 1260 liquid chromatography system. The chromatographic separation column used in this study was Poroshell 120 (Agilent Technologies, USA) with SB-C18 column (2.7 μm and 2.1 × 100 mm). A sample solution of 10 μL was injected. We detected flavonols and flavones with 0.3 mL/min of flow rate at 350 nm. The mobile phases consisted of 2.5% (v/v) A (formic acid in water) and 2.5% (v/v) B (formic acid in acetonitrile). The column temperature was kept at 30 °C with a linear gradient as follows: 0 min, 100% A; 3 min, 90% A; 5 min, 86.5% A; 7 min, 86.5% A; 8 min, 86% A; 9 min, 85% A; 10 min, 85% A; 11 min, 84.5% A; 12 min, 83.5% A; 12.5 min, 82% A; 13 min, 70% A; 13.5 min, 69.5% A; 14 min, 68.5% A; 17 min, 60% A; 20 min, 0% A; 23 min, 95% A; 25 min, 100% A. MS conditions consisted of electrospray ionization, positive ion mode; 15 psi nebulizer pressure; 300 ℃ gas (N2) temperature; capillary exit, 200 V; 4000 V capillary voltage. We collected and recorded mass spectra in range of *m*/*z* 100 to 1000. The LC/MSD-Trap™ software (version 5.3) was used to analyze these results. Kaempferol, quercetin, quercetin 3-O-rutinoside, dihydrokaempferol, dihydroquercetin were purchased from NIFDC. Quercetin 3-O-rutinoside was used as quantification reference standard. The values were expressed with mean ± SE of three replicates.

### 2.3. RNA extraction, cDNA library preparation

Total RNA of sample extract was isolated by using an improved method called CTAB (cetyltrimethylammonium ammonium bromide). To remove genomic DNA, the total RNA from each replicate was processed by Takara RNAse-free DNAse I (Dalian, China), then by using 1.0% agarose gel electrophoresis to evaluate quality and purity with RNA 6000 Nano LabChip Kitits. The cDNA library was created by reverse-transcribing cleaved RNA fragments. A total of six libraries were constructed, in which CK-1, CK-2, and CK-3 were from fruits (F), and T1-1, T1-2 and T1-3 were from YAL.

The Illumina HiSeq™ 4000 platform was performed to generate paired-end reads and analyse transcriptome of six tissues. The sequences of the 'CK' and 'T' have been deposited in the GenBank database (Accession SRP143402).

### 2.4. Data assembly, annotation, and identification of differentially expressed genes

Raw sequencing data were first filtered by deleting reads with adapter or ploy-N, as well as the ones containing over 50% nucleotide identity with a Phred quality score < 5, then unique consensus contigs were assembled using Trinity software. The longest sequences were defined as unigenes. BlastX ananlysis was performed against four protein databases, including Swissprot, KEGG (Kyoto encyclopedia of genes and genomes),Nr (NCBI non-redundant protein), and KOG (euKaryotic Ortholog Groups), by E-value < $10^{-5}$ to annotate *A. arguta* transcriptome. Based on best alignment results, functional annotations of proteins were determined. We evaluated the gene expression by FPKM method, that was fragments per kilobase of transcript per million mapped reads and performed genes analysis using KEGG and enrichment analysis of GO (gene ontology) after selecting genes of fold change >2 and FDR < 0.05 as significant ones.

### 2.5. Gene expression analysis

Eleven genes identified by transcriptome data were selected randomly to evaluate the expression level by qRT-PCR. The cDNA of each sample was compounded by TaKaRa PrimeScript RT Reagent Kit. Primers were listed in (Table S1) and devised by Primer 3.0 software. qRT-PCR reactions were conducted by a Bio-Rad Real-Time Detection System (IQ5, USA) and Takara SYBR Green PCR Master Mix. The mixtures (10 μL final volume ) consisted of 5 μL of 2 × SYBR Green Master Mix, cDNA template (1 μL), 0.5 μL of each primer (10 μmol/μL) and 2.5 μL ddH2O. The expression level in the different tissues was computed by using 2- ΔΔCt method (Δ Ct = $Ct_{sample} - Ct_{Actin}$). All the data were presented as mean ± SE.

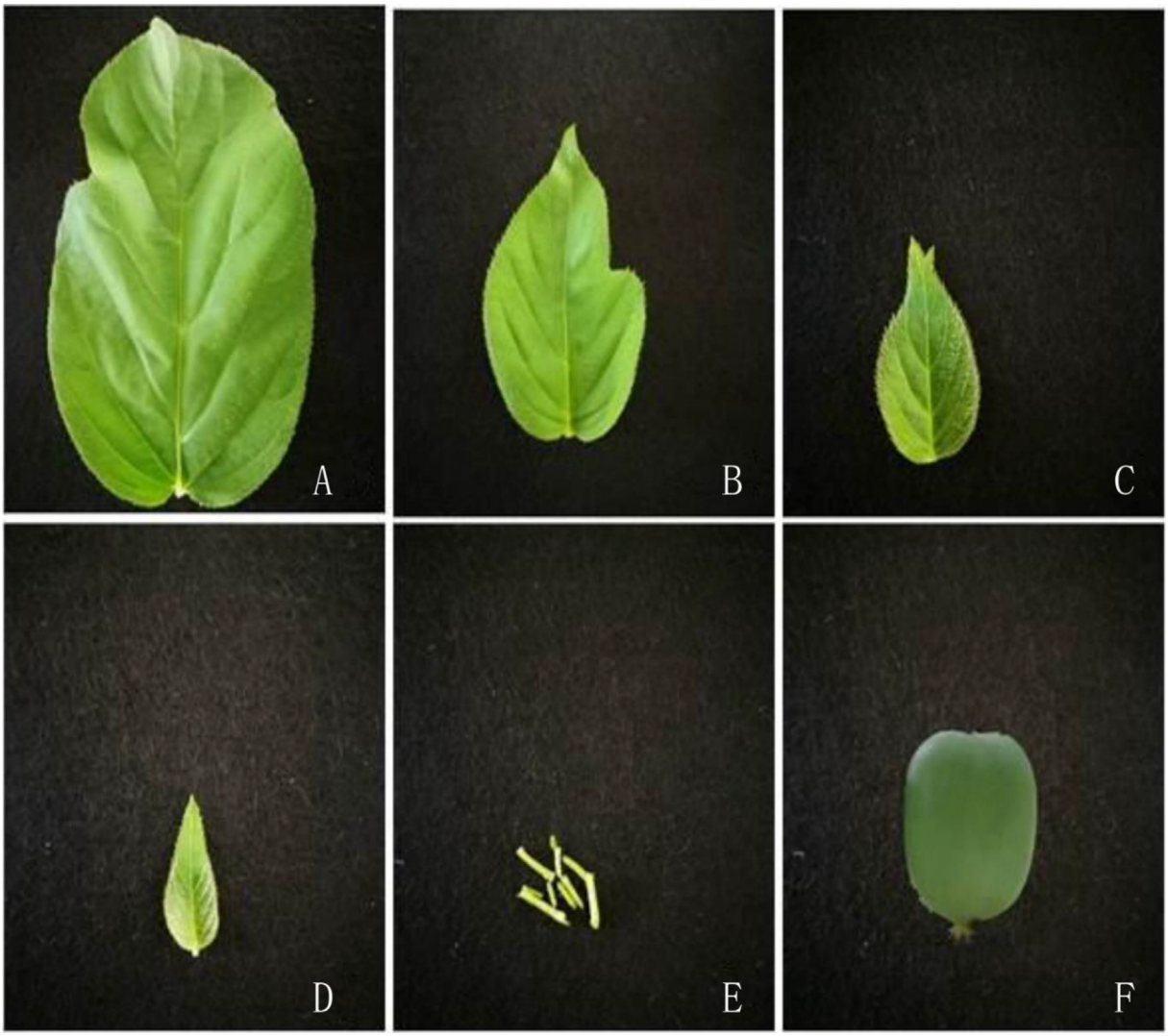


**Fig. 1.** Leaves and fruit of *Actinidia arguta* var. *arguta* cv. Changjiang No. 1. (A) Mature leaves (ML); (B) Leaves expanded to 1/2 to 2/3 of the full leaf size (HL); (C) Leaves expanded to 1/4 to 1/3 of the full leaf size (QL); (D) Young apical leaves (YAL); (E) Petioles of young apical leaves (P); (F) Fruits (F).

### 2.6. Statistical analysis

Data was analyzed with SPSS 19.0 statistically and the results represented significantly at $P < 0.05$ with the mean ± standard error.

## 3. Results

### 3.1. Analysis of 10 components of flavonoid glycosides in A. arguta

According to LC-UV-MS analysis and reference with published data, we could determine flavonoids in *A. arguta* by elution order, retention time, MS fragmentation pattern, and the UV–vis spectrum (Xue et al., 2016). The HPLC chromatogram of flavonoid compounds was detected at 350 nm (Fig. S1) and ten peaks in the flavonoid extract of *A. arguta* were detected in total. As shown in Table 1, there were some $[M + H]^+$ ions observed in mass spectrum which were strong, such as *m/z* 595, *m/z* 579, *m/z* 653, *m/z* 625, *m/z* 757, etc., also including stronger $Y_0^+$ ions and $[Y_0 + H]^-$ ions, like *m/z* 301, *m/z* 315, *m/z* 285, *m/z* 316, *m/z* 300, *m/z* 314 plasma and *m/z* 255, *m/z* 243, *m/z* 271, *m/z* 227 and some other fragment ions. Flavonol glycoside compounds were preliminarily determined to be isorhamnetin, kaempferol and quercetin. On the basis of molecular ion, main $MS^2$ fragments, the UV-vis spectrum and retention time, ten compounds were tentatively established as kaempferol-3-*O*-rutinoside (cis), kaempferol-3-*O*-rutinoside (trans), kaempferol-3-*O*- neohesperidoside (P), isorhamnetin-3-*O*-α-L-rhamnosyl-(1 → 3) -α-L-rhamnosyl-(1 → 6)-*β*-D-galactoside, Isorhamnetin-3-*O*-neohesperidoside (P) (cis), Isorhamnetin-3-*O*-neohesperidoside (P) (trans), Isorhamnetin-3-*O*-rutinoside, Isorhamnetin-3-*O*-neohespeidoside and quercetin-3-*O*-rhamnoglycoside, respectively. (Fig. S2).

### 3.2. Quantification of bioactive compounds in CJ-1

The content of flavonoids was analyzed quantitatively in different tissues of CJ-1 (Table 2). We detected ten flavonoid compounds. Quercetin-3-O-rhamnoside had the highest content. Quercetin, kaempferol and isorhamnetin were all detected in fruits, leaves and petioles. The highest concentration of flavonoid was found in leaves, far higher than those of in F and P and F, only 78.75 $\mu g \cdot g^{-1}$ and 151.63 $\mu g \cdot g^{-1}$, respectively. The highest total flavonoid content in YAL was found, which was 10219.84 $\mu g \cdot g^{-1}$, which was 67.4-fold and 129.8-fold as compared to P and F (Fig. 2). Quercetin compounds were all detected in different tissues of CJ-1 (Table 2).

**Table 1**
Mass spectraldata in leaves of CJ-1.

| Constituent | Molecular ion/$[M+H]^+$ | λmax in the visible region (nm) | Retention time/min | Fragment ions/(*m*/*z*) | M.W. (Da) |
|---|---|---|---|---|---|
| Kaempferol-3-O-Rutinoside (+) | 595 | 266,350 | 12.188 | 287 | 594 |
| Kaempferol-3-O-Rutinoside (-) | 595 | 266,350 | 13.276 | 287 | 594 |
| Kaempferol-3-O- Neohesperidoside (P) | 595 | 267,354 | 13.693 | 287 | 594 |
| Isorhamnetin-3-O-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside | 757 | 256,356 | 10.772 | 317 | 756 |
| Isorhamnetin-3-O-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside | 757 | 257,358 | 11.339 | 317 | 756 |
| Isorhamnetin-3-O-neohesperidoside (P) (+) | 625 | 266,350 | 14.363 | 317 | 624 |
| Isorhamnetin-3-O-neohesperidoside (P) (-) | 625 | 254,358 | 14.602 | 317 | 624 |
| Isorhamnetin-3-O-rutinoside | 625 | 263,356 | 14.915 | 317 | 624 |
| Isorhamnetin-3-O-neohespeidoside | 653 | 259,357 | 14.609 | 317 | 652 |
| Quercetin -3-O- rhamnoglycoside | 579 | 260,359 | 21.799 | 301 | 578 |

**Table 2**
Ten flavonoids content in different tissues.

| Flavonoids | YAL (μg·g$^{-1}$) | QL (μg·g$^{-1}$) | HL (μg·g$^{-1}$) | ML (μg·g$^{-1}$) | P (μg·g$^{-1}$) | F (μg·g$^{-1}$) |
|---|---|---|---|---|---|---|
| Kaempferol-3-O-Rutinoside (+) | 121.19 ± 8.53 f | — | 397.11 ± 24.59 c | 7.36 ± 0.52 d | — | — |
| Kaempferol-3-O-Rutinoside (−) | 775.95 ± 45.80 c | 456.45 ± 28.86 cd | — | 14.33 ± 1.07 cd | 0.56 ± 0.03 e | — |
| Kaempferol-3-O-Neohesperidoside (P) | 1999.91 ± 125.80 b | 1326.07 ± 82.84 b | 990.58 ± 59.92 a | 24.05 ± 2.19 c | 10.68 ± 0.62 cd | 3.23 ± 0.19 c |
| Isorhamnetin-3-O-α-L-rhamnopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside | 572.36 ± 34.78 cd | 499.85 ± 29.61c | 1101.83 ± 70.97 a | 56.44 ± 4.58b | 13.53 ± 0.81c | 5.65 ± 0.33c |
| Isorhamnetin-3-O-α-L-rhamnopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)-β-D-galactopyranoside | 401.43 ± 24.92 def | 323.55 ± 20.53 de | 697.98 ± 43.18 b | 57.35 ± 4.03 b | 1.32 ± 0.01 e | 0.95 ± 0.05 c |
| Isorhamnetin-3-O-neohesperidoside (P)(+) | 212.87 ± 13.58 ef | — | 28.36 ± 1.89 d | 8.59 ± 0.59 d | — | — |
| Isorhamnetin-3-O-neohesperidoside (P)(−) | 2103.58 ± 133.21b | 592.69 ± 36.25c | 115.43 ± 9.24 d | 53.39 ± 3.59b | 30.46 ± 1.75b | 12.36 ± 0.74b |
| Isorhamnetin-3-O-rutinoside | 446.09 ± 31.49 de | 210.33 ± 13.04 ef | 62.35 ± 4.23 d | 11.27 ± 0.93 d | 5.69 ± 0.32 de | — |
| Isorhamnetin-3-O-neohespeidoside | 252.63 ± 16.26 ef | 89.95 ± 5.65f | — | 7.05 ± 0.45 d | — | — |
| Quercetin −3-O- rhamnoglycoside | 3333.83 ± 221.73 a | 1478.94 ± 86.92a | 593.31 ± 44.91b | 110.72 ± 7.69 a | 89.38 ± 5.16 a | 56.55 ± 3.32 a |
| TF contents (μg·g$^{-1}$) | 10219.84 ± 1136.36 | 4977.83 ± 526.03 | 3986.94 ± 448.49 | 350.55 ± 44.39 | 151.63 ± 15.07 | 78.75 ± 8.04 |

Note: YAL, Young apical leaves; QL, Leaves expanded to 1/4 to 1/3 of the full leaf size; HL, Leaves expanded to 1/2 to 2/3 of the full leaf size; ML, Mature leaves; P, Petioles of young apical leaves; F, Fruits; — means failure to be detected.

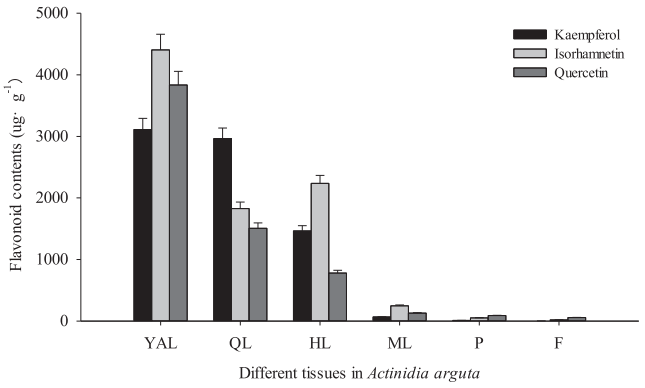


**Fig. 2.** Content of main flavonoids in different tissues of Changjiang No. 1. YAL, Young apical leaves; QL, Leaves expanded to 1/4 to 1/3 of the full leaf size; HL, Leaves expanded to 1/2 to 2/3 of the full leaf size; ML, Mature leaves; P, Petioles of young apical leaves; F, Fruits.

**Table 3**
Annotation of unigenes.

| Annotation database | Number of Unigenes | Percentage (%) |
|---|---|---|
| Annotated in NR | 36 431 | 51.58 |
| Annotated in SwissProt | 26 069 | 36.91 |
| Annotated in KOG | 21 866 | 30.96 |
| Annotated in KEGG | 14 385 | 20.37 |
| Annotated in all Databases | 11 011 | 15.59 |
| Annotated in at least one Database | 36 638 | 51.87 |
| Total Unigenes | 70 631 | 100.00 |

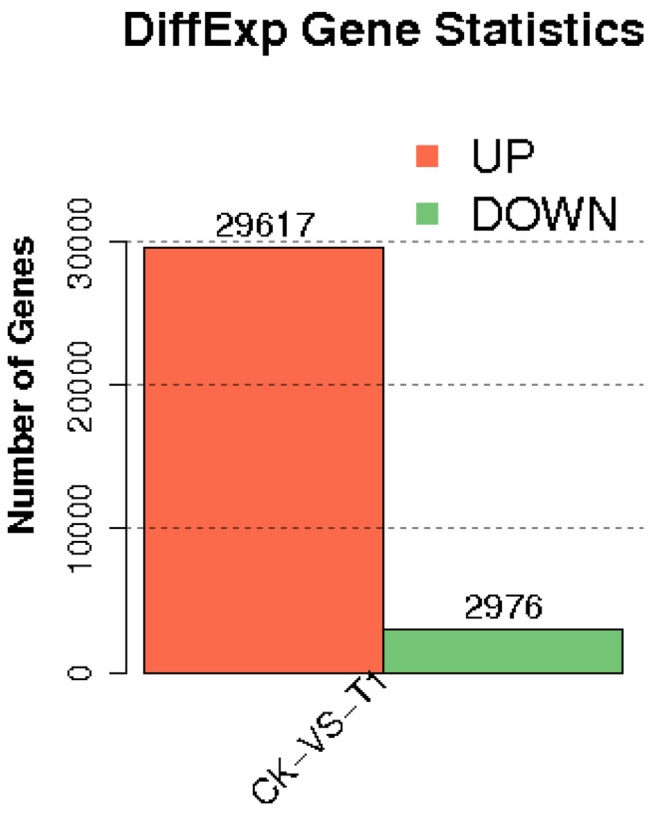


**Fig. 3.** Differential gene expression statistics.

However, their concentrations of three flavonoids were different in different tissues.

### 3.3. Transcriptome sequencing and assembly

Illumina HiSeqTM 4000 was used to carry out high throughput sequencing of six libraries. Transcriptome sequencing and assembly results were summarized in Table S3. We obtained average 7.5 Gb clean bases in total. The Q30 and GC ratio of each sample on average was 92.73% and 47.11%, respectively (Table S3). The distributions of unigenes length in CJ-1 were shown in Table S5. By sequencing and mapping alignment, it was identified overall 70,631 genes, whose average length was 798 bp (Table S4).

### 3.4. Functional annotation and identification of differentially expressed genes

The assembled unigenes were blasted against databases of SwissProt, Nr, KOG, and KEGG (E-value $\leq$ 0.00001) (Table 3). Results showed that the number annotated in all databases was 11,011 (15.59%) among 70,631 unigenes, and 36,638 (51.87%) unigenes were matched in at least one database. We identified 32,593 DEGs in total, in which 29,617 upregulated and 2976 downregulated genes in F vs YAL (Fig. 3). Then, we used GO function enrichment and the KEGG pathway to analyze DEGs. There were 12,639 DEGs to obtain from 45 significantly enriched GO terms belonging to three classifications, such as biological process (BP), molecular function (MF) and cellular component (CC) (Fig. S3).

To better understand the functional roles of DEGs in different tissues, we used the KEGG database to analyze their metabolic pathways. There were 127 metabolic pathways obtained with 3567 DEGs. The top 20 enriched pathways were listed in Fig. 4. The most significantly enriched pathway was 'plant-pathogen interaction' shown in Table S5 (Padj $\leq$ 0.05). Secondly, 'Plant hormone signal transduction' pathway was also significantly enriched with 239 DEGs, so we thought that during the leaf and fruit development, hormones may exert key regulation roles. Furthermore, 36 DEGs were enriched in 'Flavonoid biosynthesis' pathway, indicating that the synthesis of flavonoids was different in leaf and fruit, which is also the point of this study.

### 3.5. Exploring genes related to flavonoids biosynthesis

To obtain as much genetic information as possible about the flavonoid formation in *A. arguta* var. *arguta*, the core genes for all the secondary metabolic pathways including the biosynthesis of flavo-

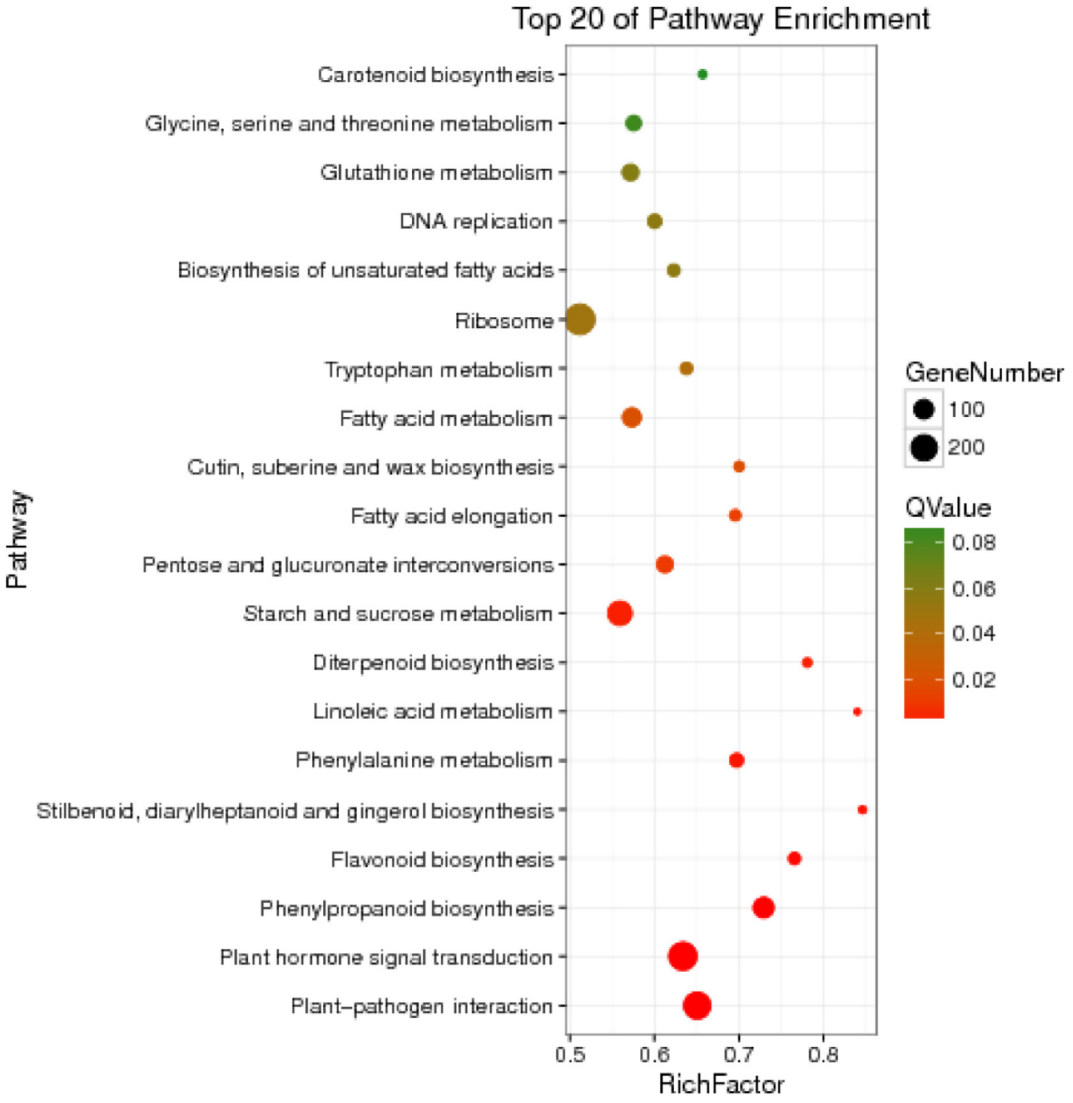


**Fig. 4.** The top 20 of most significant enrichment in KEGG pathways.

**Table 4**
Candidate genes related to flavonoid of *Actinidia arguta*.

| Function | Gene | Enzyme | KO id (EC-No.) | All[a] | Up[b] | Down[c] |
|---|---|---|---|---|---|---|
| Flavonoid biosynthesis | C4H | trans-cinnamate 4-monooxygenase | K00487 (1.14.13.11) | 1 | 1 | 0 |
| | HCT | shikimate O-hydroxycinnamoyltransferase | K13065 (2.3.1.133) | 7 | 6 | 1 |
| | C3′H | coumaroylquinate(coumaroylshikimate) 3′-monooxygenase | K09754 (1.14.13.36) | 4 | 4 | 0 |
| | CCoAOMT | caffeoyl-CoA O-methyltransferase | K00588 (2.1.1.104) | 3 | 3 | 0 |
| | CHS | chalcone synthase | K00660 (2.3.1.74) | 3 | 3 | 0 |
| | CHI | chalcone isomerase | K01859 (5.5.1.6) | 1 | 1 | 0 |
| | F3H | naringenin 3-dioxygenase | K00475 (1.14.11.9) | 3 | 3 | 0 |
| | F3′H | flavonoid 3′-monooxygenase | K05280 (1.14.13.21) | 1 | 1 | 0 |
| | F3′5′H | flavonoid 3′,5′-hydroxylase | K13083 (1.14.13.88) | 3 | 3 | 0 |
| | FLS | flavonol synthase | K05278 (1.14.11.23) | 2 | 2 | 0 |
| | DFR | bifunctional dihydroflavonol 4-reductase/ flavanone 4-reductase | K13082 (1.1.1.219/ 1.1.1.234) | 3 | 3 | 0 |
| | ANS | anthocyanidin synthase | K05277 (1.14.11.19) | 1 | 1 | 0 |
| | LAR | leucoanthocyanidin reductase | K13081 (1.17.1.3) | 3 | 3 | 0 |
| | ANR | anthocyanidin reductase | K08695 (1.3.1.77) | 1 | 1 | 0 |
| Anthocyanin biosynthesis | UFGT75C1 | anthocyanidin 3-O-glucoside 5-O-glucosyltransferase | K12338 (2.4.1.298) | 3 | 1 | 2 |
| | UGAT | cyanidin-3-O-glucoside 2″-O-glucuronosyltransferase | K12937 (2.4.1.254) | 4 | 4 | 0 |
| Flavone and flavonol biosynthesis | kaempferol 3-O-beta-D-galactosyltransferase | kaempferol 3-O-beta-D-galactosyltransferase | K13269 (2.4.1.234) | 1 | 1 | 0 |
| | AOMT | flavonoid O-methyltransferase | K13272 (2.1.1.267) | 1 | 1 | 0 |

Note: All[a] indicates the total number of unigenes analysed, Up[b] indicates the number of unigenes with expression significantly up-regulated in leaves than that in fruits, Down[c] indicates the number of unigenes with expression significantly down-regulated in leaves than that in fruits.

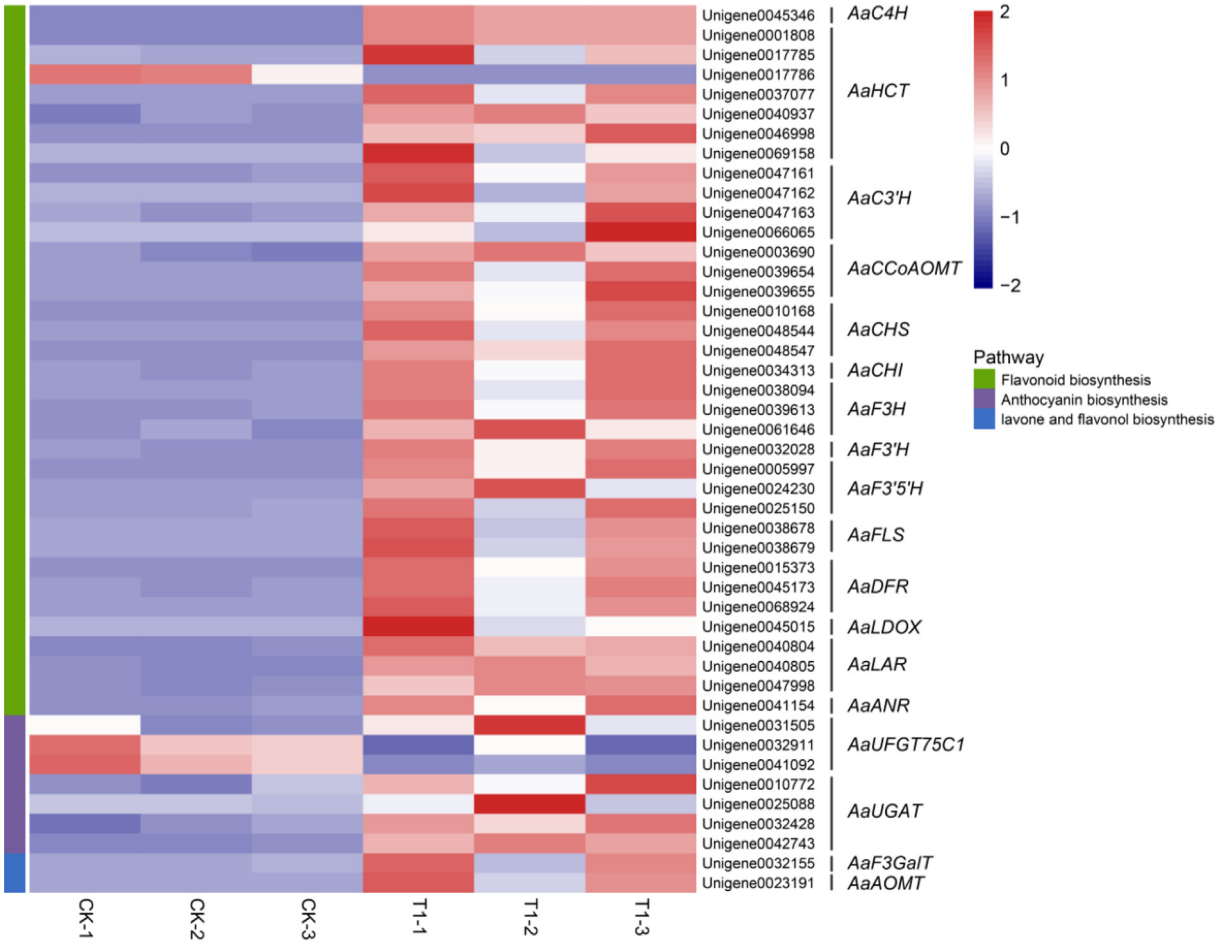


**Fig. 5.** The heatmap was conducted according to the Z-score normalized data of expression profile of flavonoids formation related genes.

noid, anthocyanin, flavone, and flavonol, were extracted from the transcriptional database. Based on the KEGG database, a comprehensive search was carried out in the annotated results of CJ-1. Then according to the corresponding KO ID of the annotated results (Table 4), the corresponding gene was found according to the reference path provided in the KEGG database. There were 45 DEGs identified in total, which were connected with the biosynthesis of flavonoid, flavone and flavonol, and anthocyanin. All these DEGs indicated differential expressions in leaves and fruits, and most of them were up-regulated in leaves.

Ten DEGs were annotated that encoded four enzymes in the upstream biosynthesis of flavonoid, which were all up-regulated in leaves than in fruits, including chalcone synthase (CHS, 3 DEGs), flavonoid 3′-hydroxylase (F3'H, 1 DEG), flavonoid 3′, 5′-hydroxylase (F3'5'H, 3 DEGs) and flavanone 3-hydroxylase (F3H, 3 DEGs). While in the downstream step, five DEGs were encoding two enzymes, all expressed in leaves predominantly, which were dihydroflavonol 4-reductase (DFR, 3 DEGs) and flavonol synthase (FLS, 2 DEGs). We thought that these two enzymes played a central role in flavonoid formation in leaves. In addition, we also anno-

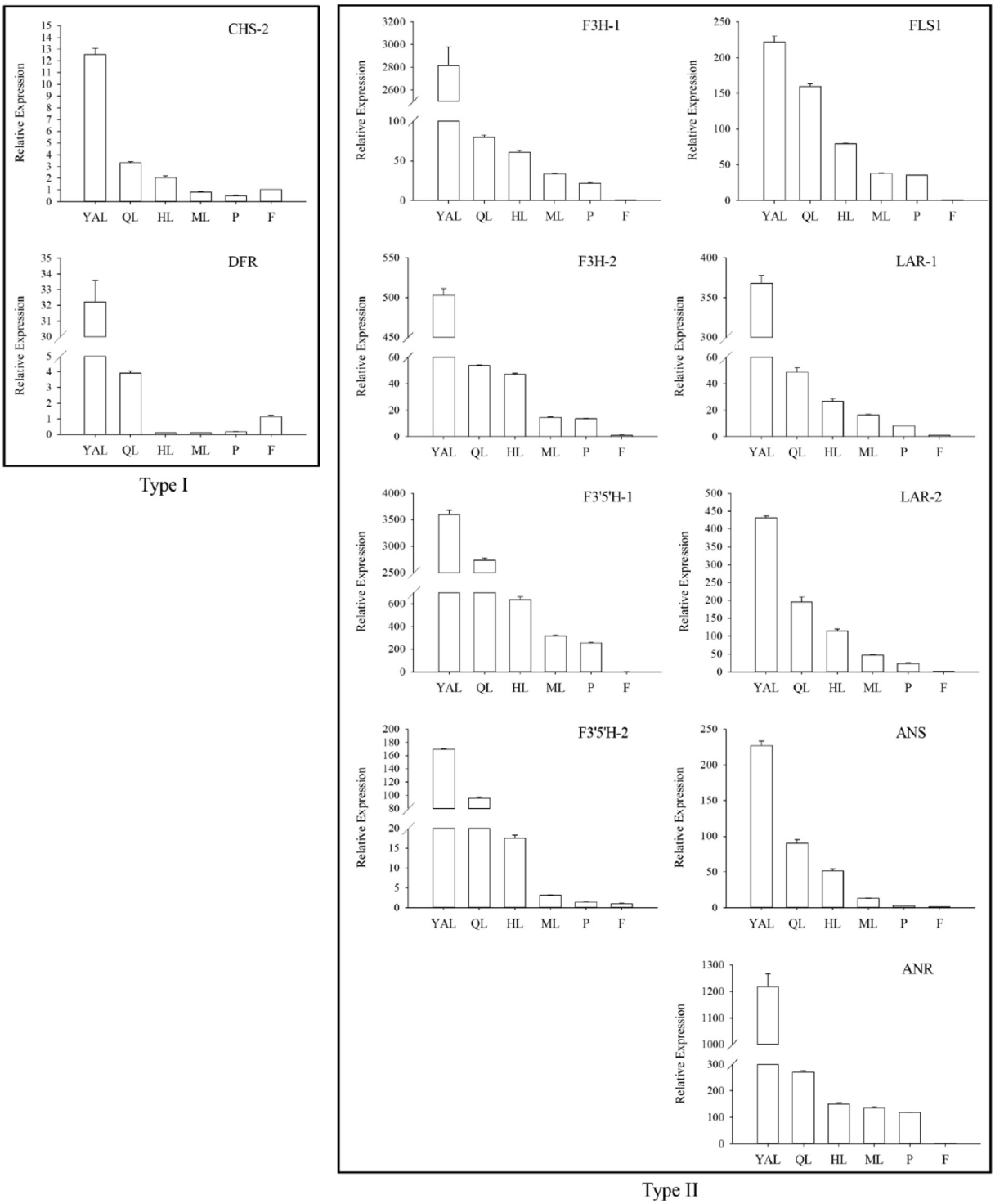


**Fig. 6.** Gene expression associated with flavonoid synthesis by qRT-PCR in different tissues.

tated four DEGs, encoding anthocyanidin synthase (ANS, 1 DEG), leucoanthocyanidin reductase (LAR, 3 DEGs) and anthocyanidin reductase (ANR, 1 DEG), respectively. To further learn the roles of genes associated with flavonoids formation in leaves and fruits, a heatmap was plotted (Fig. 5). DEGs all showed different expression in different tissues. By analyzing the pathway significant enrichment of differentially expressed genes, almost all the enzymes were annotated in the flavonoid biosynthesis pathway, indicating that the sequencing of the transcriptome of F and YAL in CJ-1 contained large amounts of information.

### 3.6. Expression of gene-related with the flavonoid biosynthesis

To confirm the unigenes obtained from transcriptome data, eleven key genes were examined in flavonoid biosynthesis with qRT-PCR. It confirmed that the expression patterns of eleven genes in fruit and leaves were similar to the transcriptome. The expression profiles of these genes were shown in Fig. 6. On the whole, key genes expression in leaves was higher than in other tissues in flavonoid biosynthesis, like *AaF3'5'H-1 and AaF3H-1*. These genes were partitioned to two types in the light of the expression pattern, those of one type lowered gradually with the developing of leaf, such as *AaF3H-1*, *AaFLS-1*, *AaF3'5'H-1*, *AaANS*, *AaLAR-1*, etc. While the other type only included two genes of *AaDFR* and *AaCHS*, which expressed higher in F than in ML and P, lower than in other tissues.

## 4. Discussion

One of the most important secondary metabolism pathways is the flavonoid biosynthetic pathway. For many years, many researchers have been studying deeply on the biosynthesis and regulation of flavonoids, and it has been reported in strawberry (Markus et al., 2008), onion (Park et al., 2017), and *Ginkgo biloba* (Wu et al., 2018). In *a citrus* study, transcript levels for *CHS*, *CHI*, and *F3H*, as well as flavonoid contents, were all high in young tissues, decreasing with the ripening, which indicated that flavonoid synthesis began from the early development (Moriguchi et al., 2002). Moreover, in this study, the presented above core gene expression data were consistent with the data of transcriptome sequencing and flavonoid quantification. More expression in young leaves, and concentration of flavonoids in young apical leaves was more than 100 times that of fruits. It was consistent with the working hypothesis of Gutierrez et al. (2017) that flavonols accumulated only in leaves and there was a strong flavonols synthesis in leaves. Similarly, the expression of *AaFLS*, *AaF3'5'H* and *AaF3H* also highly in YAL in this study, which were the core genes synthesizing isorhamnetin, quercetin and kaempferol. We inferred that higher content of quercetin in YAL was caused of the highest expression of *AaFLS* and *AaF3'5'H* gene in YAL, then acting together on dihydrokaempferol to synthesize more quercetin. As we know, isorhamnetin was synthesized by benzylation and methylation of quercetin. Therefore, it was believed that the key regulatory genes of the isorhamnetin metabolic pathway were *AaFLS* and *AaF3'5'H*, which also caused the highest isorhamnetin content in YAL.

In some crop studies, it had been confirmed that *F3'5'H* and *F3'H* genes were involved in regulating the content and synthesis of flavonoids, such as kiwifruit (Peng et al., 2019), melon, etc. It had also been verified that *DFR* gene could directly catalyze producting naringenin from pelargonidin (Jin et al., 2016). In *Solanum lycopersicum* research, Payyavula et al. (2013) reported that the expression of most phenylpropane genes decreased with the development of tubers. This study found that *AaANR* gene also expressed highest in YAL, which may be related to the *AaDFR* gene and *AaF3H* gene expressing highly in upstream. In addition, the high expression of the three genes AaDFR, *AaANS* and *AaLAR* also resulted in the highest total flavonoid content in YAL.

It was reported that flavonoids from the plant could restrain free radical reactions propagating to protect from diseases, they could also control the growth of the plant and single organ (Meng et al., 2019). Simultaneously, the development of natural antioxidants of plant origin has been devoted more considerable regard (Khoobi et al., 2011). *A. arguta* var. *arguta* is a precious wild resource native to China. For many years, it has been commercially cultivated rapidly in China. So far, some studies on the extraction, purification, and identification of flavonoids from *A. arguta* have been reported (Latocha and Jankowski, 2011; Wojdyło et al., 2017). It was indicated in this study that the flavonoids in *A. arguta* young leaves belonged to isorhamnetin, quercetin and kaempferol, furthermore, the content of total flavonoids was higher as compared to mature leaves. Guo et al. (2020) informed that more active flavonoid metabolism was in young *Ginkgo biloba* leaf. Maybe the young *A. arguta* leaves will be apllied in the fields of food processing and healthcare like *G. biloba* (Jain et al., 2011) and *Lycium barbarum* (Zhou et al., 2017).

## 5. Conclusion

In conclusion, we explored flavonoid accumulation mechanism in the *A. arguta* fruits and leaves at the metabolome and transcriptome levels. With the use of LC-MS/MS, ten flavonoids were identified in fruits and leaves, including quercetin, kaempferol and isorhamnetin, and the total flavonoid were higher in leaves than in fruits significantly. We identified 45 candidate genes involving in the biosynthesis of flavonoid, flavone and flavonol, anthocyanin. Moreover, eleven key DEGs related to flavonoid biosynthesis expressed higher in young apical leaves, consistent with the data of transcriptome sequencing and flavonoid quantification. *A. arguta* is a bioactive functional plant species and its leaves with high contents of flavonoids, which could be a promising source of leaf tea or herbal medicine. Our results contributed to further study of the complex physiological process and mechanism of flavonoid accumulation in different tissues.

## Declaration of Competing Interest

All authors state that they have no competing commercial interests or individual connections that could have affect the work informed in this study.

## Acknowledgments

The authors thank the Project of Shenyang High-end Academic Exchange Activities (2018-8) for funding this research.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jksus.2021.101605.

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