

Metabolomic and transcriptomic exploration of the Biosynthetic Pathways of Uric Acid-reducing flavonoids in the fruit of *Actinidia arguta* Sieb. Zucc.

Yubo Wang

Shenyang Agricultural University

Kuiling Dong

Mudanjiang Normal University

Minghui Zhang

Shenyang Agricultural University

Xiaojuan Yin

Shenyang Agricultural University

Chunhui Hao

Liaoning Agriculture College

Muhammad Irfan

University of Sargodha

Li-jing Chen (✉ chenlijing1997@126.com)

Shenyang Agricultural University <https://orcid.org/0000-0002-2489-9933>

Yong Wang

College of Agriculture

Research Article

Keywords: *Actinidia arguta* Sieb.Zucc., Flavonoids biosynthesis pathway, Uric acid-reducing flavonoids, Transcriptome, Widely targeted metabolomics, QRT-PCR

Posted Date: May 11th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1549931/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Extensive targeted metabolomic and transcriptomic analyses were used to determine the types of flavonoids present in the fruit of two varieties of *Actinidia arguta* Sieb. Zucc. cultivated in Northern China. Fractionation and preparative liquid chromatography were used to isolate and purify the potentially biologically active flavonoids chrysin (flavone1), rutin (flavone2), and daidzein (flavone3) from the fruit of *Actinidia arguta* Sieb. Zucc. The structures of these flavonoids were appraised using mass spectroscopy/high-performance liquid chromatography (MS-HPLC). Serum levels of uric acid (UA), urea nitrogen (BUN), creatinine (Cr), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and liver glycogen were measured in male Kunming mice treated with each flavonoid to determine biological effects of the flavonoids on the measured parameters. The three flavonoids were found to be mainly distributed within three metabolic pathways, namely the flavonoids and flavonol biosynthesis pathway (BP), the flavonoid BP, and the secondary metabolite BP. Through a correlation analysis of the transcriptome and metabolome, regulatory genes were screened for activity in the BPs of the three flavonoids. This is the first study to explore the UA-lowering activity of flavonoids in the fruit of *Actinidia arguta* Sieb., the main variety cultivated in Northern China. These data provide valuable information for understanding the biosynthetic mechanisms and regulatory pathways of flavonoids in *Actinidia arguta* Sieb. and contribute to its development as a food and drug homologous functional food.

1 Introduction

Actinidia arguta Sieb. Zucc. commonly-known as soft jujube kiwifruit, macaque pear, or rattan melon, is a perennial plant in the Actinidiaceae family. It is distributed in Jilin, Heilongjiang, Liaoning, and Shandong Provinces in North and Northwest China, and matures from June to August each year. It's one of the famous and economically important wild fruits in the Changbai Mountain Area of China; due to its green color, juiciness, and delicately sweet and sour flavor, it is known as "the precious fruit of the world" (Almeida et al. 2018; AustinG et al. 2021). *Actinidia arguta* Sieb. Zucc. is nutritionally rich and contains a variety of functional components, such as amino acids, vitamins, and flavonoids. It is an excellent raw material for the growth of functional health foods [Carla et al. 2021; Changhua et al. 2021; Chenglei et al. 2012]. Flavonoids are one of the main active components of *Actinidia arguta* Sieb. Zucc. grown in Changbai Mountain. Flavonoids have anti-oxidation and anti-viral functions, in addition to preventing cardiovascular disease, cerebrovascular disease, and hyperuricemia, and they contribute to liver protection and immunity [Chenglei et al. 2012; Gois and Souza 2020; Hang-qing et al. 2016]. *Actinidia arguta* Sieb. Zucc. has broad application prospects for the development of flavonoid products in the field of medicine and food homology. At present, the UA reducing activities and BPs of its flavonoids, chrysin, rutin, and daidzein, have not been systematically analyzed.

Bioflavonoids, most of which are light yellow to yellow, share the basic core structure of 2-phenylchromogen ketone. The term flavonoids are commonly used as the general name for compounds formed by the connection of two benzene rings through a central three-carbon chain. The biological activities of flavonoids are mainly due to the presence of α , and β -unsaturated pyranone. Substituents of

the A, B, and C rings determine the biological activities of different bioflavonoids. Natural bioflavonoids have a small molecular weight and can penetrate adipose tissue, pass through the blood-brain barrier, and are quickly absorbed by the human body, characteristics that form the material basis for the pharmacological roles played by these molecules (Jiang et al. 2020; Kateryna et al. 2020). In recent years, scholars both domestically and internationally have carried out research into the active components and functional mechanism of such botanical drugs and found that flavonoids such as morin, quercetin, luteolin, and kaempferol have significant effects in treating hyperuricemia (Hua) and gout [Latocha et al. 2014; Latocha et al. 2013]. We knew flavonoids can prevent Hua by inhibiting the activity of xanthine oxidase (XO) and by accelerating the excretion of UA. Gouty arthritis can be prevented by restraining the release of inflammatory transmitters by neutrophils and by inhibiting the expression and secretion of inflammatory cytokines, which are induced by urate crystallization [Letizia et al. 2021; Lijun et al. 2021].

Hua is a pathological state in which UA levels in the blood increase continuously or blood is supersaturated with UA. The number of patients with Hua in China exceeded 170 million in 2017, and the data show a rapid increase in cases, with an annual growth rate of 9.7%. Gender, age, race, and lifestyle habits all affect the incidence of Hua (Lijun et al. 2017; Lili et al. 2012; Ling et al. 2021; Maria Giulia et al. 2020; Mo et al. 2020). The key cause of primary Hua is a combination of low UA excretion and high UA production. A majority (67%) of UA in the human body is produced by the catabolism of nuclear proteins, nucleic acids, and other substances in the body; the remaining 33% comes from purines in food [Namll et al. 2011; Nidhi et al. 2011; Shuangshuang et al. 2021]. Adenosine deaminase (ADA) and xanthine oxidase (XO) are the key enzymes that regulate the production of UA during the catabolism of purine substances to UA. ADA is a sulfhydryl enzyme that catalyzes the reaction of adenine nucleosides to produce hypoxanthine nucleosides. Hypoxanthine is then produced through the action of nucleoside phosphorylase, and hypoxanthine is finally oxidized by the flavin protease XO to produce UA [Shuangshuang et al. 2019; Singh et al. 2015; Vernerová et al. 2021; Xiang et al. 2021; Xiaohua et al. 2012].

Broadly targeted metabolomics is a new tool for non-targeted analysis and identification of all metabolites in a sample under specific conditions (Xiaojie et al. 2015; Xiaolin and Jie 2021). Metabolomic data have been processed using multivariate analysis [Xiaoyun et al. 2019]. Multivariate statistical analyses, such as principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), can divide datasets into different groups and screen candidate metabolites for variation between samples. This research method is helpful in the identification and analysis of phytochemical characteristics; it is also used for the analysis of bioactive compounds in food science. In previous studies, we found through metabolomic analysis that phenolic compounds in chyme grains have high antioxidant capacities; metabolomics revealed the significance of specific chyme grain metabolites for the antioxidant properties and various traits. These results show that metabolomic methods are suitable for the identification of bioactive compounds in functional foods. Transcriptomics allows for the identification of genes that are differentially expressed between samples, which can then be annotated and studied for enrichment. This type of analysis is the basis for studying gene function and structure, and it is important in the study of organisms' growth and disease occurrence. Previously,

10 kinds of flavonoids belonging to quercetin, isorhamnetin, and kaempferol have been detected in the leaves of *Actinidia arguta* Sieb.Zucc. A total of 70631 unigenes were confirmed from transcriptome data, containing 29,617 up-regulated and 2976 down-regulated genes in fruits and young leaves. Genes such as *AaF3'5'h-1h* and *AaF3h-1* are more highly expressed in leaves than in other tissues [Yi-Hsien et al. 2021]. RNA-seq was conducted on the fruits of two *A. arguta* varieties showed colorless anthocyanin dioxygenase (*LDOX*) may be the key gene regulating anthocyanin synthesis in the flesh of 'tianyuanhong' (an all-red fruit variety) [Yingfeng et al. 2019]. In addition, through comparative analysis with homologous genes in *Arabidopsis thaliana*, most structural genes related to flavonoid biosynthesis in bitter mustard have been confirmed, and the functions of some have been verified [Yuanyuan et al. 2016; Yukuo et al. 2018; Zicheng et al. 2019].

In this study, the UA-lowering effect of flavonoid extract from *Actinidia arguta* Sieb. Zucc. was evaluated *in vitro*. Furthermore, the types, quantities, and differences between flavonoids in the fruits of two important varieties of *Actinidia arguta* Sieb.Zucc. cultivated in Northern China were determined through a combination of extensively targeted metabolome and transcriptome analyses. The BPs and structural genes involved in regulating the flavonoid compounds populin (flavone1), rutin (flavone2), and daidzein (flavone3), which have UA-reducing activity, were analyzed and identified. This provides valuable information for further improving the fruit quality of *Actinidia arguta* Sieb.Zucc. breeding new varieties, and developing food and drug homologous functional foods from *Actinidia arguta* Sieb.Zucc.

2 Materials And Methods

2.1 Plant materials and sampling

Mature fruit was collected from 8-year-old *Actinidia arguta* Sieb.Zucc. varieties Qssg and LC grew in Northeast, North, and Northwest China and Shandong Province. Fresh fruit samples were stored at -80°C until further use.

All the chemicals such as 95% ethanol, petroleum ether, n-butanol, rutin standard, HPD600 macroporous adsorption resin, and absolute ethanol were of analytical grade. Allopurinol sustained-release capsule, purchased from Heilongjiang aolidanede Pharmaceutical Co., Ltd; Ethambutol hydrochloride tablets, purchased from Hangzhou Minsheng Pharmaceutical Co., Ltd; Adenine, purchased from American sigma company; Acetaminophen sustained-release tablets, purchased from Shanghai Johnson & Johnson Pharmaceutical Co., Ltd; UA kit, bun (urea nitrogen) kit, Cr (creatinine) kit, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) kit and glycogen kit were purchased from Quanzhou konodi Biotechnology Co., Ltd.

Kunming white mouse, weighing 19-21g, purchased from Shenyang Changsheng biology Co., Ltd. Before starting the experiment, they were settled to environmental adaption for at least one week. 6 animals per cage (320× 180 × 160 cm), according to the normal 12 hour / 12 hour light and dark schedule. Temperature: 22 ± 2 °C; Relative humidity: 55 ± 5% and standard food and water were given during the

study. All experiments were conducted according to the requirements of the institutional animal care committee of Nanjing University and the China Animal Care Council of Nanjing University [SYSK (SU) 2009–0017].

2.2 Composition analysis of flavonoids in two kinds of *Actinidia arguta* Sieb.Zucc.

Total flavonoids were measured in the fruit samples. Metware Biotechnology Co., Ltd. (Wuhan, China) conducted 785 metabolite analyses. In short, cryopreserved samples at ultra-low temperatures were freeze-dried and ground with a grinder until it was a powder (Mixer Mill MM 400, Retsch, Haan, Germany) at 30 Hz for 1.5 min. Next, 100 mg of powder was mixed with 1.2 ml of 70% methanol. Samples were vortexed once every 30 minutes for 30 seconds a total of six times, then incubated overnight at 4°C. After centrifugation at 12,000 rpm for 10 min, retain the filtrate with a microporous membrane. An ultra-performance liquid chromatography (UPLC)-tandem mass spectroscopy (MS/MS) system was used to analyze the samples. Flavonoids were identified using a local database. A multiple reaction monitoring (MRM) model was used to quantitatively analyze the flavonoids (Dong, et al, 2019; Dong, et al, 2014; Chen, et al, 2013). Flavonoids were further annotated online with PlantCyc (<http://www.plantcyc.org/>) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.kegg.jp/>). PCA and OPLS-DA were used to determine differentially accumulated flavonoids, where $|\log_2(\text{fold change})| \geq 1$ was used as the threshold.

2.3 Flavonoid isolation, identification, and UA-lowering activity test

Frozen *Actinidia arguta* Sieb.Zucc. samples were thawed, washed with distilled water, dried, sliced, and ground in a homogenizer. Crude flavonoid extract was obtained by mixing the ground tissue with ethanol, centrifuging, filtering, and concentrating samples. A rutin standard curve was generated to determine the concentration of flavonoids in the crude extract. Macroporous resin and preparative liquid chromatography were then used for separation and purification. Electrospray ionization mass spectrometry (ESI-MS) was applied to identify flavonoids.

Thirty-six male Kunming mice were randomly separated into six groups after seven days of adaptive feeding in a clean, quiet environment at $22 \pm 2^\circ\text{C}$ and relative humidity of $55 \pm 5\%$. The experimental groups were as follows: blank control group, model control group, positive control group (allopurinol), aspen (flavone1), rutin (flavone2), and daidzein (flavone3). The blank group was gavaged with distilled water, and the animals in all other groups were gavaged with a 2.5% Papa suspension for seven consecutive days. On the eighth day, the mouse model of hyperuricemia was induced by gavage with 100 mg/kg adenine and 250 mg/kg ethambutol. Animals in the treatment groups (i.e., groups other than the blank and model controls) were fed the same amount of distilled water, and the appropriate drugs were administered by gavage. The dose of flavonoids was 550 mg/kg. The dosage of allopurinol tablets was 33.3 mg/kg (administration volume: 1 ml/100 g) for 23 consecutive days.

One hour after treatment administration on the 7th and 15th days, eyeball blood was collected. The serum was centrifuged and serum levels of UA, urea nitrogen (BUN), creatinine (Cr), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and hepatic glycogen were measured using kits.

2.4 RNA extraction, library construction, and sequencing

An EasySpin Plus Plant RNA Kit (Aidlab) was used to obtain total RNA from frozen fruits, which was then treated with DNase I (Takara) to clear it of contaminating DNA. mRNA was cleaned from the total RNA with a Dynabeads mRNA Purification Kit (Invitrogen, Waltham, MA, USA). The resulting purified mRNA was fragmented, after which first-strand cDNA was synthesized using random hexamer primers, and second-strand cDNA was synthesized with a NEBNext Ultra RNA Library Preparation Kit (New England Biolabs, Ipswich, MA, USA). cDNA was purified, ends were repaired, and the fragments were ligated to adapters. cDNA was then fragmented again after which it was enriched via PCR and the last cDNA library was built for Illumina paired-end sequencing. Biomarker Technology Co., Ltd. (Beijing, China) sequenced the cDNA library using the HiSeq Xten PE150 platform (Illumina, San Diego, CA, USA).

2.5 RNA sequencing data analysis

Adapter sequences and low-quality reads were removed to obtain clean reads, which we then mapped to the reference gene sequence (<http://www.org/pinkul>). Unigenes were determined using hisat2 and stringtie. Gene functional annotation was performed using BLAST to search several databases, namely the NCBI non-redundant (NR) database, Swiss-Prot, Gene Ontology (GO), Clusters of Orthologous Genes (COG), Eukaryotic Orthologous Genes (KOG), Pfam, and KEGG, using an e-value cutoff of 10^{-5} . The expression level for each gene was determined and then normalized according to the number of fragments per kilobase of transcript per million mapped reads (FPKM).

The deseq software package was applied to identify DEGs between samples. False discovery rate (FDR) < 0.05 and $|\log_2(\text{fold change})| \geq 1$ were used as thresholds for defining DEGs. Functional enrichment analysis was performed on the DEGs using GO annotations and KEGG pathways.

2.6 qRT-PCR analysis

qRT-PCR was used to prove the RNA-Seq data and measure the expression of structural genes bound up with flavonoid biosynthesis. qRT-PCR analysis was conducted according to previously outlined methods (Tan et al, 2021; Park, et al, 2011).

2.7 Correlation analysis between metabolites and transcripts

The correlation analysis was carried out by identifying the correlation coefficient of the content of flavonoids and the transcriptional changes in both differentially expressed flavonoids and differentially expressed genes. Both of them are rich in flavonoids and flavonol BP (ko00941), flavonoid BP (ko00942), and secondary metabolite BP (ko00943). To analyze the BP and structural genes related to the regulation

of flavonoids with UA reducing activity, such as populin (flavone1), rutin (flavone2), and daidzein (flavone3).

3 Results

3.1 Flavonoid composition and UA-reducing capacity of two varieties of *Actinidia arguta* Sieb.Zucc.

To study the composition of flavonoids during the fruit ripening stage of *Actinidia arguta* Sieb.Zucc., widely targeted metabolomics was used for the first time. LC-ESI-QTrap-MS/MS and multiple reaction monitoring were used to generate multi-peak maps. Specific ions for each substance were identified with the triple four-stage rod, while the signal strength (CPS) of the characteristic ions was generated using a detector. We opened the sample off-line mass spectrometry file using the multiquant software to conduct qualitative and quantitative analyses of the metabolite spectrum in Qssg and LC *Actinidia arguta* Sieb.Zucc.(Fig. 1).

There are 125 flavonoids were identified, including 18 flavones, 6 dihydroflavonols, 12 Dihydroflavones, 12 flavonols, 64 flavonols, two chalcone compounds, three flavonoid carboglycosides, one isoflavone, and 7 procyanidins (Table 1). PCA highlighted that these two varieties were significantly different; 68.61% of the differences between the samples could be explained by PC1 (40.7%) and PC2 (27.9%), revealing a change in the pattern of flavonoid accumulation between the two varieties. Hierarchical cluster analysis (HCA) showed that most of the detected flavonoids changed greatly in concentration during fruit ripening, consistent with the differences in PCA results between the two varieties (Fig. 2). Both Qssg and LC fruits contain flavonoids, such as populin (flavone1), rutin (flavone2), and daidzein (flavone3), which were isolated and purified respectively. The UA lowering activity test was carried out through the male Kunming mouse animal model(Fig. 2). Compared with the model control group, the three selected flavonoids significantly reduced UA after one week ($p < 0.01$) (Fig. 2C). At two weeks, flavone3 treatment significantly reduced UA ($p < 0.05$), but there was no remarkable difference in UA activity between flavone1/flavone2 and the control group. Flavone3 had relatively stable biological activity in reducing UA. Compared with the blank control group, BUN was not remarkably increased in the model control group (Fig. 2D). Compared with the model control group, only flavone3 significantly reduced BUN ($p < 0.05$), and there was no remarkable difference in the BUN-reducing activity of the other two flavonoids. Thus, flavone3 had a higher biological capacity to reduce BUN than flavone1 or flavone2 did. Flavone3 also significantly reduced Cr compared to the model control group ($p < 0.05$), but there was no remarkable difference in Cr reducing activity between the other two flavone treatment groups (Fig. 2E). Compared with the model control group, flavone2, flavone3, and the positive control group (treated with allopurinol) all had significantly lower levels of GAPDH activity ($p < 0.01$), meaning that only the flavone1 treatment group showed no significant difference (Fig. 2F). Flavone3 also significantly increased liver glycogen compared to the model control group ($p < 0.01$); in contrast, flavone2 remarkably decreased liver glycogen ($p < 0.01$), and there was no remarkable difference in liver glycogen in those treated with flavone1 (Fig. 2G).

3.2 Differences of flavonoids in Qssg and LC fruits, enrichment analysis of KEGG pathway, and structural verification results.

OPLS-DA can maximize the distinction among categories and identify differentially accumulated metabolites. It uses orthogonal signal correction (OSC) and PLS-DA techniques to identify variable differences. The abundance of the 118 flavonoids was compared between samples using the OPLS-DA model (Fig. 4). LC samples were found to the left of the confidence interval, while the Qssg samples were found to the right of the confidence interval, clearly demonstrating the ability of the model to discriminate between the two varieties.

We generated two principal components from OPLS-DA; the contribution rate was 62.1% for principal component 1 and 9.74% for principal component 2 ($R^2x = 0.807$, $R^2y = 0.998$ [$p = 0.29$], $Q^2 = 0.869$ [$p < 0.005$]). The OPLS-DA results were verified by performing 200 replicates of the analysis. Model verification (Fig. 5) demonstrated that the OPLS-DA model was biologically meaningful and superior to the PCA model. The differential flavonoids can be screened according to VIP analysis.

The pathway enrichment analysis of flavonoids was carried out through KEGG database. The results indicated that the 125 identified flavonoids were mainly distributed in three metabolic pathways,(1) flavonoid and flavonol BPs, primarily for kaempferol-3-o-neohesperidin Luteolin-7-o-neohesperidin, quercetin-3-o-sangbu diglycoside, quercetin-3-o-(2"-o-xylosyl)-rutoside, and kaempferol-3-o-rutoside;(2) flavonoid BPs, mainly including naringin-7-o-glucoside, isolygeride, and chrysin;(3) secondary metabolite BPs, primarily kaempferol-3-o-rutoside and daidzein(Fig. 3).

In a mass spectrum graph, the mass charge ratio (M/z) of the ion increases from left to right, and the abscissa of an ion with a single charge is the mass of its ion; the ordinate represents the intensity of the ion current, usually expressed in terms of relative intensity. The elution rate is 68.8% for 50% ethanol, 51.2% for 60% ethanol, and 48.2% for 70% ethanol. Based on our test data, the peak value of flavonoids eluted by 50% ethanol was more and the elution rate was higher. Therefore, only positive ion mode mass spectra of flavonoids eluted by 50% ethanol are discussed here. The mass spectrum information for full scanning mode was m/z 609, 301, and 175, the multi-reaction detection mode was m/z [609/301], and the neutral loss was 308u. By comparing the retention times, multi-stage mass spectrum fragments, excimer ion peaks, and other information with reference substances, we determined that the molecular ion peak was m/z 609 for rutin, m/z 254 for chrysin, and m/z 254 for soybean isoflavone (Figure).

3.3 Transcriptome analysis of two *Actinidia arguta* Sieb.Zucc. Varieties.

To further study potential molecular mechanisms of flavonoid biosynthesis in *Actinidia arguta* Sieb.Zucc., nine cDNA libraries were constructed for high-throughput RNA-Seq analysis. Each library obtained 281,758,296 reads from 439,776,908 to 43,580,384, and each library obtained 269,732,354 clean reads from 41,844,326 to 41,708,692 (Table 2). The q30 percentage (which includes sequences having an error rate $< 0.1\%$) for each library exceeded 91%, and the mean GC content was 47.47%. Among

the clean reads, between 78.54% and 80.76% could be mapped to the reference genome. A total of 43,686 unique genes were identified. The analyses suggest high-quality RNA-Seq data suitable for use in further analyses.

3.4 Differential gene expression in two *Actinidia arguta* Sieb.Zucc. Varieties.

DEGs in the mature fruits of two *Actinidia arguta* Sieb.Zucc. varieties were identified by first analyzing the correlation coefficient between the profile clustering of gene expression and biological replicates (Fig. 4). This analysis indicated that many genes exhibited differential expression in various samples. Correlation coefficients for the levels of gene expression of the biological replicates were > 0.8 , indicating that the biological replicates were consistent with one another and the data were therefore valid for the identification of DEGs. Using threshold values of $FDR < 0.05$ and $|\log_2(\text{fold change})| \geq 1$, DEGs between the two varieties were identified; there were 6497 up-regulated and 5153 down-regulated genes compared between Qssg and Lc (Fig. 5).

To comprehend the biological functions of DEGs, we used Blast GO to determine enriched GO terms. Among the 48 enriched functional groups, there were 25 biological processes, 13 cell components, and 10 molecular functions (Fig. 6 and table 3). Among the biological processes, the most frequent annotations were metabolic processes, cellular processes, and single biological processes. For cell component annotations, the most common terms were organelle, cell part, and cell. For molecular function annotations, the most represented terms were binding, catalytic activity, and transporter activity.

KEGG analysis was conducted to identify enriched metabolic pathways in the DEGs. There were 4024 DEGs distributed among 142 KEGG pathways (Table 4). Among them, there were 42 remarkably enriched pathways ($p\text{-value} < 0.05$) (Table 5). Flavonoid biosynthesis (ko00941), flavonoid and flavonol biosynthesis (ko00944), and secondary metabolite biosynthesis (ko01110) pathways were all enriched, and the flavonoid biosynthesis (ko00941) and secondary metabolite biosynthesis (ko01110) pathways were significantly enriched. Flavonoid biosynthesis (ko00941) and secondary metabolite biosynthesis (ko01110) exist in the pathway of significant enrichment (Fig. 7). All of the enriched pathways could be further divided into five categories: cellular processes, genetic information processing, environmental information, metabolism, and tissue systems. Among these categories, the metabolism category contained the most pathways (Fig. 12a-c), and the highest numbers of DEGs were bound up with amino acid biosynthesis (ko01230; 185 genes), carbon metabolism (ko01200; 190 genes), and sucrose and starch metabolism (ko00500; 207 genes).

3.5 Analysis of flavonoid biosynthesis and regulation of differential gene expression in two kinds of *Actinidia arguta* Sieb.Zucc. at maturity.

The enriched KEGG pathways and gene functional annotations were used to determine which DEGs encode flavonoid biosynthesis-related enzymes, flavonoid, and flavonol biosynthesis-related enzymes, and secondary metabolite biosynthesis-related enzymes. In total, 41 were found, including six CsUGT134

genes, seventeen LOC, two AT2, one CHS, one CHI, three C4H, one HCT, one CCoAOMT, one F3H, two CFOL, one DFR, three LAR2, one LSAT, and one GSCOC (Table 6). Among those, six UGT9491, sixteen LOC, two AT2, one CHS, two C4H, one HCT, one CCoAOMT, one F3H, two LAR2, and one GSCOC were significantly up-regulated in the two varieties. Two CFOL, one CHI, one C4H, one DFR, one LAR2, one LOC, and one LSAT were significantly down-regulated between the two varieties.

3.6 qRT PCR was used to confirm the results of DEGs bound up with flavonoid biosynthesis.

To validate the expression of the DEGs bound up with flavonoid biosynthesis, qRT-PCR was performed for 11 structural genes (one CsUGT134, one LAR2, one C4H, one CFOL, one CHI, two LOC, one GSCOC, one CCoAOMT, one DFR, and one AT2) in the fruits of the two varieties at maturity. The selected genes were highly correlated between RNA-Seq and qPCR data, effectively validating the transcriptome data (Fig. 8).

3.7 Correlation between transcripts and flavonoid derivatives.

To comprehend the pathway and regulatory structural genes of flavonoids biosynthesis in the fruits of two main varieties of *Actinidia arguta* Sieb.Zucc., the quantitative changes of flavonoids and transcripts in the fruit ripening stage of *Actinidia arguta* Sieb.Zucc. was tested and analyzed by studying the interaction between transcriptome and metabolome. Based on the results of DEGs and Dems rich in flavonoid BP, it is annotated that 20 structural genes and regulatory groups show a higher correlation with the BP of flavonoid compounds aspen (flavone1), rutin (flavone2) and daidzein (flavone3) (Table 7). Their interaction network is shown in Fig. 9.

Analysis results: according to the analysis in the pathway diagram in Fig. 9a, DFR, F3'H, and FLS compete with the substrate dihydrokaempferol DHK. According to the gene expression analysis in Fig. 9C, FLS has relatively high expression and high activity of its coding enzyme, which finally promotes the synthesis of rutin. It is also found in Fig. 9b that the relative content of rutin accumulated in the sample is high; The expression of LOC was relatively low, and the relative contents of populin and daidzein were relatively low.

4 Discussion

Flavonoids are substances that have been studied in-depth and at length in traditional Chinese medicine. They exist widely throughout the kingdom Plantae. Many studies have been absorbed on the abilities of flavonoids to reduce blood lipid levels, inhibit lipid peroxidation, relieve coughs, eliminate phlegm and asthma, and exert anti-tumor, anti-hepatotoxicity, anti-inflammatory, analgesic, antibacterial, and antispasmodic effects. Substances known to reduce UA include quercetin, luteolin, apigenin, puerarin, catechins, and dyestuffs, but there have been few studies into the UA-lowering activities of aspen and daidzein.

In this experiment, extensive targeted metabolomics methods were used to compare the flavonoids present in the fruit of two varieties of *Actinidia arguta* Sieb. Zucc. cultivated in Northern China. Nine classes and 125 kinds of flavonoids were detected in the fruits of the two varieties; these included 39 kinds of differentially accumulated flavonoids, accounting for 31.2% of the total flavonoids detected. This proved that there was a remarkable distinction in flavonoid composition and accumulation between different varieties. The types and proportions of different flavonoids found in the fruits were as follows: 51.2% flavonols, 14.4% flavonoids, 9.6% dihydroflavonoids, 9.6% flavonols, 1.6% chalcones, 2.4% flavone carboglycosides, 4.8% dihydroflavonols, 0.8% isoflavones, and 5.6% procyanidins. The relative flavonoid content in the fruits of variety Qssg was higher (1.13×) than in LC variety fruits. These data provide a valuable theoretical and practical basis for breeding new varieties of *Actinidia arguta* Sieb. Zucc. and developing food and drug homologous functional foods.

The flavonoids rutin, populin, and daidzein were isolated from the fruit of the main variety of *Actinidia arguta* Sieb. Zucc. cultivated in Northern China and tested for their UA-reducing activity. The results showed that populin (flavone1), rutin (flavone2), and daidzein (flavone3) could reduce serum levels of UA, BUN, Cr, and GAPDH in mice to varying degrees. We, therefore, speculate that UA production in mice (in response to purine-rich food ingestion or catabolism of substances such as nuclear proteins and nucleic acids) may be inhibited by reducing the activities of ADA and/or XO. Increases in liver glycogen content in mice may be due to inhibition of glycolysis by flavonoids through the promotion of gluconeogenesis, reduction of free glucose in the blood, promotion of the remedial synthesis pathway of nucleotides, and decreases in serum UA content. This study, therefore, has strong practical significance for the prevention and control of human Hua through the comprehensive development of *Actinidia arguta* Sieb. Zucc. as a treatment.

Through pathway enrichment analysis of flavonoids in *Actinidia arguta* Sieb. Zucc., we found that the 125 flavonoids identified in the fruits were mainly distributed in three metabolic pathways: the flavonoid and flavonol BP, the flavonoid BP, and the secondary metabolite BP. This finding provides a strong basis for the observed enrichment of flavonoids in *Actinidia arguta* Sieb. Zucc.

There are two general types of genes bound up with the biosynthesis of plant flavonoids [44]. The first category is structural genes, which encode enzymes that catalyze the biosynthesis of different flavonoids; the second category is regulatory genes, which control the transcription of structural genes [33,35]. Through transcriptome analysis, 43,686 genes were identified as related to flavonoid biosynthesis in the fruits of two main varieties of *Actinidia arguta* Sieb. Zucc, including 11,650 differentially expressed genes. These genes regulate metabolism, cellular processes, genetic information processing, environmental information, and tissue systems in the fruits of *Actinidia arguta* Sieb. Zucc. Through KEGG enrichment analysis and gene functional annotation, 41 DEGs encoding enzymes known to be involved in flavonoid biosynthesis were identified (containing CsUGT134, LOC, AT2, CHS, CHI, C4Ha, HCT, CCoAOMT, F3H, CFOL, DFR, LAR2, LSAT, and GSCOC). Among these DEGs, three CsUGT 134 genes, one LAR2, one C4Ha, two CFOL, one CHI, nine LOC, one GSCOC, one CCoAOMT, one DFR, and two AT2 genes are crucial to flavonoid biosynthesis.

A correlation analysis between transcriptome and metabolome profiles showed that expression levels of some structural genes were closely bound up with the accumulation of specific flavonoids, demonstrating that expression of these flavonoid biosynthesis genes promoted flavonoid accumulation during fruit ripening of the two main varieties of *Actinidia arguta* Sieb. Zucc. CHS, CHI, F3H, cfol, LOC, LSAT, fnsi, DFR, F3'H, FLS, and Hidh may play structural or regulatory roles in the BPs of the flavonoid compounds aspen (flavone1), rutin (flavone2), and/or daidzein (flavone3). DFR, F3'H, and FLS compete with the substrate dihydrokaempferol (DHK); FLS expression was relatively high, and the encoded enzyme activity was high, which ultimately promotes rutin accumulation. LOC expression was relatively low, as were the relative levels of populin and daidzein.

In conclusion, these results expand our understanding of Hua prevention and control by flavonoids. The results also demonstrate the mechanisms of flavonoid biosynthesis and accumulation in *Actinidia arguta* Sieb. Zucc.. In total, these data provide precious information for the future development of novel food and drug homologous functional foods using *Actinidia arguta* Sieb. Zucc..

Declarations

Corresponding Author

Lijing Chen – Key Laboratory of Agriculture Biotechnology, College of Biosciences and Biotechnology, Shenyang Agricultural University, Liaoning 110866, China ✉orcid.org/0000-0002-2489-9933; Phone: +86-024-88490005; E-mail: chenlijing1997@126.com , chenlijing@syau.edu.cn; Fax: +86-024-88492799

Yong Wang – Liaoning University of science and technology, Liaoning, 110325, China ✉E-mail: wy2020866@126.com

Authors

Yubo Wang – Key Laboratory of Agriculture Biotechnology, College of Biosciences and Biotechnology, Shenyang Agricultural University, Liaoning 110866, China ✉E-mail: wangyubo@stu.syau.edu.cn

Kuiling Dong – Oriental Language Institute, Mudanjiang Normal University, Mudanjiang 157011, China ✉E-mail: dongkuiling@163.com

Minghui Zhang – Key Laboratory of Agriculture Biotechnology, College of Biosciences and Biotechnology, Shenyang Agricultural University, Liaoning 110866, China ✉E-mail: zhangminghui0509@126.com

Xiaojuan Yin – Key Laboratory of Agriculture Biotechnology, College of Biosciences and Biotechnology, Shenyang Agricultural University, Liaoning 110866, China ✉E-mail: yinxiaojuan43@126.com

Chunhui Hao – Liaoning University of science and technology, Liaoning, 110325, China ✉E-mail: 19044433@qq.com

Acknowledgements This work was financially supported by Scientific research projects of Heilongjiang Provincial Education Department(Grant No.1354MSYYB012) and scientific research project of Mudanjiang Normal University,(Grant No.YB2020014.)

Author contributions

LJC and YW designed and drafted the manuscript. YBW performed experiments and analyzed the data. YBW wrote the first draft of the manuscript with the help of KLD and YW. XJY has been involved in partial data analysis and figure compile and edit.MHZ, CHH and M I helped to review and edit the manuscript. LJC has been involved in critically revising the manuscript for important intellectual content; All authors gave the final approval of the version to be published.

Conflict of interest The authors declare no conflict of interest

References

1. Almeida D, Pinto D, Santos J, Vinha A.F, Palmeira J, Ferreira HN, Rodrigues F, Oliveira MBPP (2018) Hardy kiwifruit leaves (*Actinidia arguta*): an extraordinary source of value-added compounds for food industry. *Food Chem* 259:113-121
2. Austin G S, Nalina D, Joanna P, Susanne J, Eva J, Fredrik E, Robert T (2021) Effect of Intensive Urate Lowering With Combined Verinurad and Febuxostat on Albuminuria in Patients With Type 2 Diabetes: A Randomized Trial. *Am J Kidney Dis* 77(4):481-489
3. Carla P, Pedro C, Larindja P, Victor MB, Adelaide A (2021) Kiwifruit bacterial canker:an integrative view focused on biocontrol strategies. *Planta* 253(2):49
4. Changhua T, Zhigang W, Xiuli F, Baitao P, Muhammad I, Changjiang L (2021) Transcriptomic and metabolomics of flavonoid compounds in *Actinidia arguta* var.*arguta*. *J King Saud Univ Sci* 33:2-9
5. Chenglei L, Yuechen B, Hui C, Haixia Z, Jirong S, Qi W (2012) Cloning, characterization and Functional analysis of a phenylalanine ammonia-lyase gene (FtPAL) from *Fagopyrum tataricum* Gaertn. *Plant Mol Biol Rep* 30:1172-1182
6. Chenglei L, Yuechen B, Shuangjiang L, Hui C, Xueyi H, Haixia Z, Jirong S, Sang-Un P, Qi W (2012) Cloning, characterization and activity analysis of a flavonol synthase gene FtFLSI and its association with flavonoid content in tartary buckwheat. *J Agric Food Chem* 60:5161-5168
7. Gois PHF, Souza ERM (2020) Pharmacotherapy for hyperuricaemia in hypertensive patients. *Cochrane Database Syst Rev* 9:CD008652(1-32)
8. Hang-qing C, Cai-ying P, Ying-zheng H, Ying G, Jian-qun L, Rui Z, Ji-cheng S (2016) Flavonoids from leaves of *Psidium littorale*. *Acta Pharmaceutica Sinica* 51(11):1745-1750

9. Jiang LL, Gong X, Ji MY, Congcong W, Li MH (2020) Bioactive Compounds from Plant-Based Functional Foods: A Promising Choice for the Prevention and Management of Hyperuricemia. *Foods* 9(973):973-997
10. Kateryna P, Aditi D, Nadiia M, Robert T, Paulina S, Eduardo S, Stefan P, Danica G (2020) Oral Treatment With an Engineered Uricase, ALLN-346, Reduces Hyperuricemia and Uricosuria in Urate Oxidase -Deficient Mice. *Front Med*. <https://doi.org/10.3389/fmed.2020.569215>
11. Latocha P, Krupa T, Radzanowska J, Jankowski (2014) Changes in postharvest physicochemical and sensory characteristics of hardy kiwifruit (*Actinidia arguta* and its hybrid) after cold storage under normal versus controlled atmosphere. *Postharvest Biol Technol* 88:21-33
12. Latocha P, Wolosiak R, Worobiej E, Krupa T (2013) Clonal differences in antioxidant activity and bioactive constituents of hardy kiwifruit (*Actinidia arguta*) and its year-to-year variability. *Sci Food Agric* 93:1412-1419
13. Letizia P, Massimo B, Maria Giulia B, Andrea B (2021) Xanthine oxidoreductase: A leading actor in cardiovascular disease drama. *Redox Bio* 148:102195(1-10)
14. Lijun L, Shenjun J, Xuqiang, L, Qi T, Yan C, Jiaojiao Q, Li W, Qiang W, Yanli W, Jinmei W, Yan Z, Wenyi K (2021) Inflammatory Response and Oxidative Stress as Mechanism of Reducing hyperuricemia of *Gardenia jasminoides* with Network Pharmacology. *Oxidative Med Cell Longev*. <https://doi.org/10.1155/2021/8031319>
15. Lijun Z, Xiuxiu L, Bin M, Qiang G, Huilong D, Yuanhuai H, Yan L, Yinghao C, Ming Q, Yaxin Z, Hongwei L, Mingchuan M, Longlong L, Jianping Z, Chenghu N, Yongjun Q, Jun W, Lin C, Huimin L, Chengzhi L, Zhijun Q (2017) The Tartary buckwheat genome provides insights into rutin biosynthesis and abiotic stress tolerance. *Mol Plant* 10:1224-1237
16. Lili Z, Zhenyu W, Ziluan F, Shuangqi T, Jiaren L (2012) Evaluation of Antioxidant and Antiproliferative Properties of Three *Actinidia* (*Actinidia kolomikta*, *Actinidia chinensis*) Extracts in Vitro. *Int J Mol Sci* 13:5508-5511
17. Ling C, Tianyi Z, Yu X, Luan X, Yueying C, Feng Q, Yu X, Weiguo W, Man H, Quan J, Liwei L, Hejian Z, Xiaoxia Z (2021) The Anti-Inflammatory and UA Lowering Effects of Si-Miao-San on Gout. *Front. Immunol*. <https://doi.org/10.3389/fimmu.2021.777522>
18. Maria Giulia B, Massimo B, Andrea B, Letizia P (2020) Pro-Aging Effects of Xanthine Oxidoreductase Products *Antioxidants* 09(9):839(1-16)
19. Mo C, Chengyi Y, Jianing Z, Peiyu Z, Yujie J, Xiaoyong L, Huaxiang W (2020) Bergenin as a Novel Urate-Lowering Therapeutic Strategy for hyperuricemia. *Front Cell Dev Biol*. <https://doi.org/10.3389/fcell.2020.00703>
20. Namll P, Xiaohua L, Tatsuro S, SunJu K, SunHee W, CheolHo P, SangUn P (2011) Differential expression of anthocyanin biosynthetic genes and anthocyanin accumulation in tartary buckwheat cultivars 'Hokkai T8' and 'Hokkai T10'. *Agric Food Chem* 59:2356-2361
21. Nidhi G, Sunil KS, Jai CR, Rajinder SC (2011) Expression of flavonoid biosynthesis genes vis-a-vis rutin content variation in different growth stages of *fagopyrum* species. *Plant Physiol* 168:2117-2123

22. Shuangshuang L, Guanhua X, Junyu L, Liyan W, Heng C, Jin L (2021) The Role of Advanced Imaging in Gout Management. *Front Immunol*. <https://doi.org/10.3389/fimmu.2021.811323>
23. Shuangshuang Z, Yong W, Jinsong C, Ninghuang F, Ruochi Z, Zhenyu X, Fuxing Z, Wenyan Z, Dandan Z (2019) Hyperuricemia and Cardiovascular Disease. *Curr Pharm Des* 25(6):700-709
24. Singh PR, Kumar VA, Sandeep A, Sharma AK, Virendra K, Sanjeev A (2015) Physicochemical and antioxidant properties of kiwifruit as a function of cultivar and fruit harvested month. *Braz Arch Biol Techn* 58(2):6-9
25. Vernerová A, Kujovská Krčmová L, Melichar B, Švec F (2021) Non-invasive determination of UA in human saliva in the diagnosis of serious disorders. *Clin Chem and Lab Med* 59(5):797-812
26. Xiang OY, Nazhi L, Minxia G, Manman Z, Jie C, Litao Y, Jixiao Z (2021) Active flavonoids from attenuate monosodium urate-induced gouty arthritis inhibiting TLR4/MyD88/NF- κ B pathway and NLRP3 expression. *Front. Pharmacol*. <https://doi.org/10.3389/fphar.2021.760331>
27. Xiaohua L, Ayeaye T, Namli P, Tatsuro S, SunJu K, SangUn P (2012) Accumulation of phenylpropanoids and correlated gene expression during the development of tartary buckwheat sprouts. *Agric Food Chem* 60:5629-5635
28. Xiaojie Y, Cunshan Z, Hua Y, Xingyi H, Haile M, Xiaopei Q, Jiali H (2015) Effect of ultrasonic treatment on the degradation and inhibition cancer cell lines of polysaccharides from *Porphyra yezoensis*. *Carbohydr Polym* 117:650-656
29. Xiaolin L, Jie G, Jinhui T (2021) Purinergic Signaling in the Regulation of Gout Flare and Resolution. *Front Immunol*. <https://doi.org/10.3389/fimmu.2021.785425>
30. Xiaoyun Q, Xu W, Jing L, Yao L, Juan P (2019) Hypouricemic and nephroprotective roles of anthocyanins in hyperuricemic mice. *Food Funct* 10(2):867-878
31. Yi-Hsien C, Yi-Fen C, Hsin-Yuan C, Yun-Ju H, Kai-Lee W, Yong-Han H, Mohamed A, Tzong-Ming S, Shih-Min H (2021) Anti-Inflammatory and Anti-Hyperuricemic Effects of Chrysin on a High Fructose Corn Syrup-Induced Hyperuricemia Rat Model via the Amelioration of Urate Transporter and Inhibition of NLRP3 Inflammasome Signaling Pathway. *Antioxidants* 10(4):564-578
32. Yingfeng S, Liuqing X, Min T, Lu F, Jiasun L, Hongwei G, Shuchen M, Tao L, Yi W, Weifang B, Andong Q, Shougang Z, Na L (2019) Blockade of enhancer of zeste homolog 2 alleviates renal injury associated with hyperuricemia. *Am J Physiol-Renal* 316(3):488-505
33. Yuanyuan H, Zuoxu H, Dongyang L, Xingbin Y (2016) Tartary buckwheat flavonoids protect hepatic cells against high glucose-induced oxidative stress and insulin resistance via MAPK signaling pathways. *Food Funct* 7:1523-1536
34. Yukuo L, Jinbao F, Xiujuan Q, Miaomiao L, Yunpeng Z, Leiming S (2018) A key structural gene, AaLDOX, is involved in anthocyanin biosynthesis in all red-fleshed kiwifruit (*Actinidia arguta*) based on transcriptome analysis. *Gene* 648:31-41
35. Zicheng Z, Qing Z, Yang Y, Yu W, Jiuliang Z (2019) Highly Acylated Anthocyanins from Purple Sweet Potato (*Ipomoea batatas* L.) Alleviate hyperuricemia and Kidney Inflammation in Hyperuricemic Mice: Possible Attenuation Effects on Allopurinol. *J Agric Food Chem* 67(22):6202-6211

Tables

Tables 1-7 are not available with this version.

Figures



Fig 1 Qssg and Lc of two main cultivars of *Actinidia arguta* Sieb.Zucc. in Northern China.

Figure 1

See image above for figure legend.

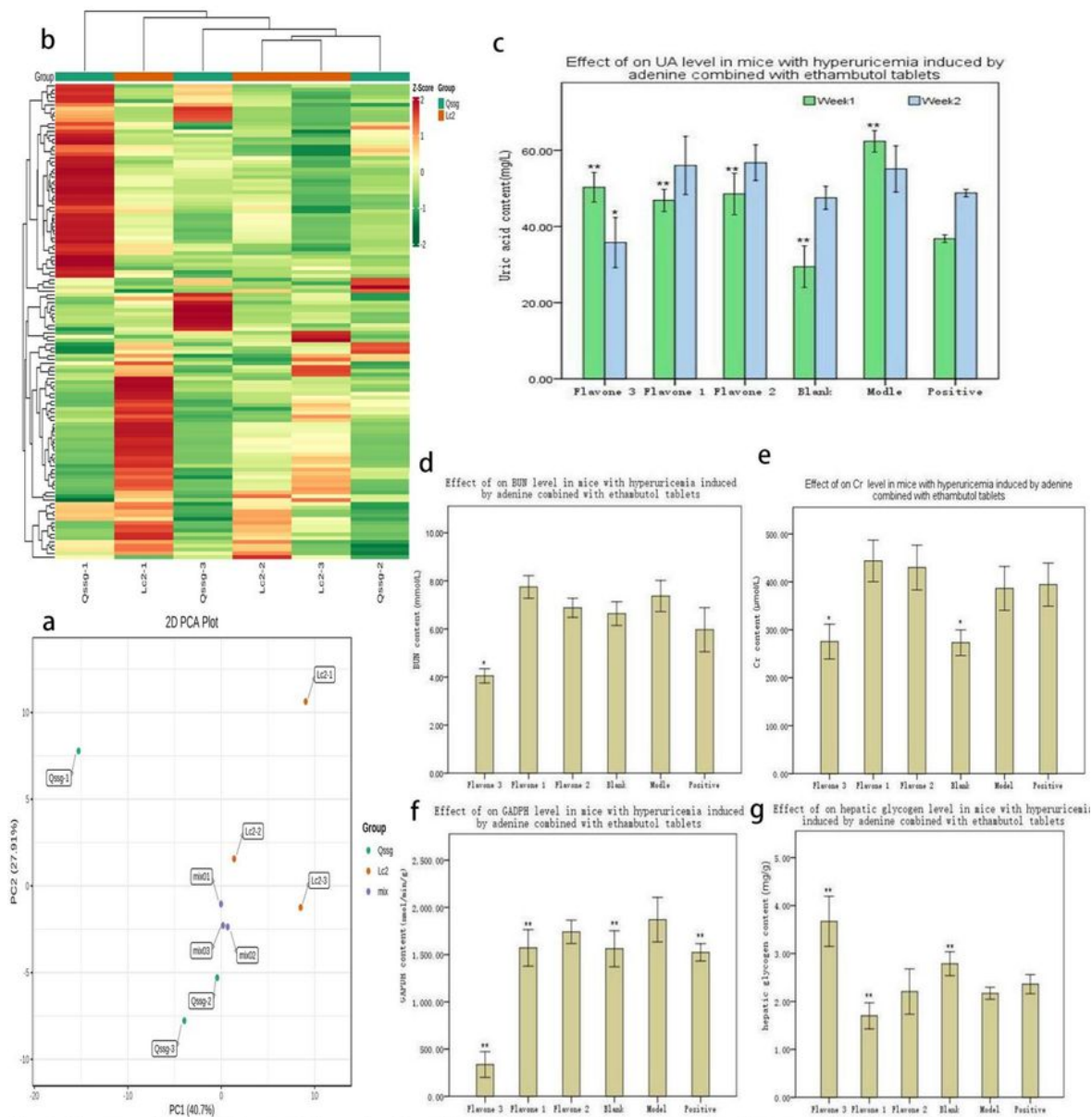


Fig. 2 Composition of flavonoids and uric acid reducing analysis results in Qssg and Lc *Actinidia arguta* Sieb. Zucc. **a** PCA score map of metabolites in Qssg and Lc fruits, each point representing an independent biological repeat. **b** Volcano map of metabolites in Qssg and Lc fruits. There are obvious differences between the two samples. **c** Effect of adenine combined with UA levels in mice with hyperuricemia. **d** Effect of adenine combined with ethambutol tablets and acetaminophen on BUN levels in mice with hyperuricemia. **e** Effect of adenine combined with ethambutol tablets acetaminophen on Cr level in mice with hyperuricemia. **f** Effect of adenine combined with ethambutol GAPDH levels in mice with Hyperuricemia. **g** Effect of adenine combined with tablets and acetaminophen on hepatic glycogen levels in mice with hyperuricemia.

Figure 2

See image above for figure legend.

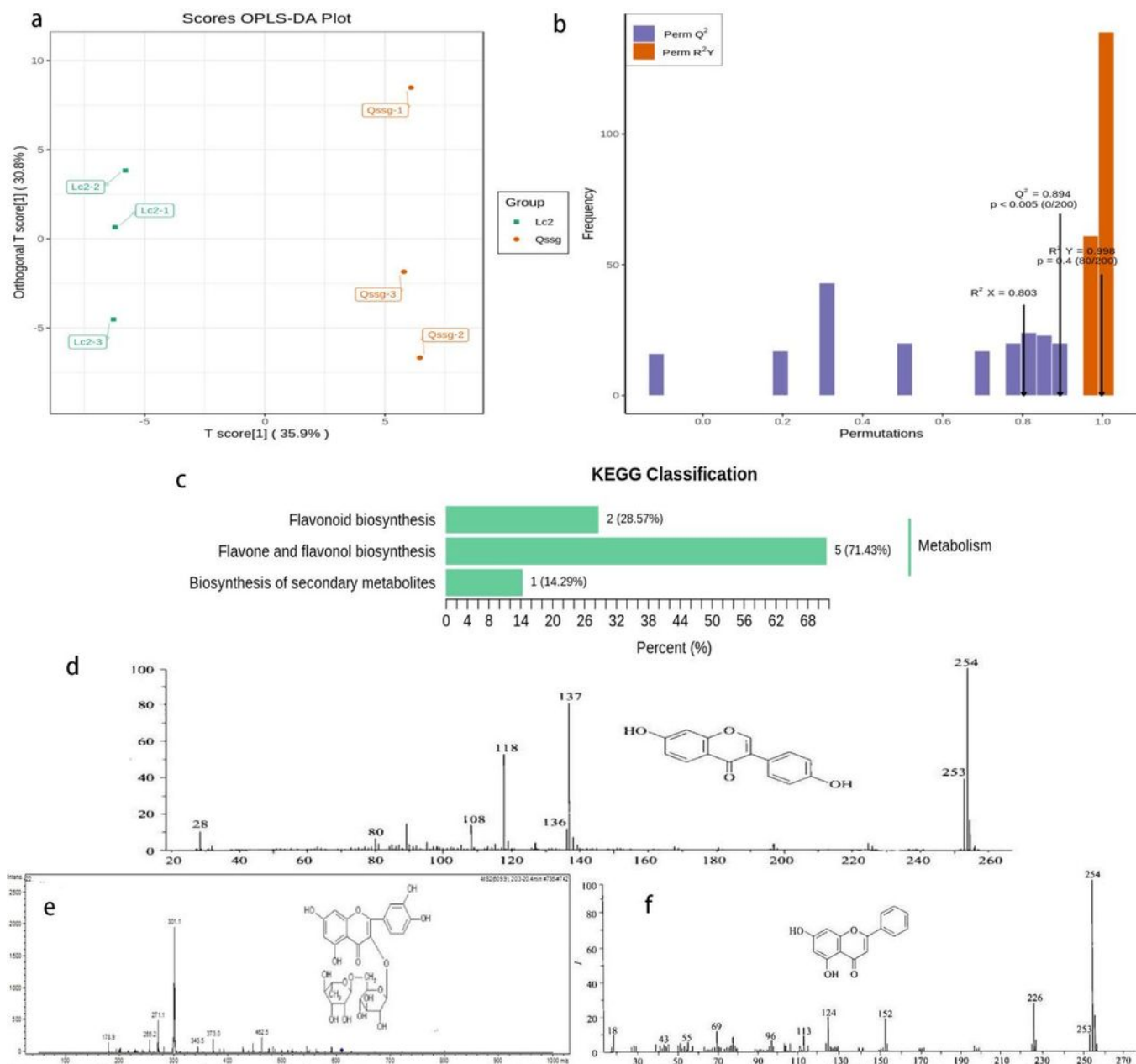


Fig. 3 Difference of flavonoids in Qssg and Lc *Actinidia arguta* Sieb. Zucc., enrichment analysis results of KEGG pathway and structure diagram of rutin, chrysin and Daidzein. **a** Opls-da score map of metabolites in Qssg and Lc fruits, and each point represents an independent biological repeat. **b** Opls-da model validation diagram of metabolites in Qssg and Lc fruits ($P < 0.005$). **c** KEGG classification map of differential metabolites in Qssg and Lc fruits. **d** Rutin ionization and mass spectrum. **e** Ionization and mass spectrum of populin. **f** Daidzein ionization and mass spectrometry.

Figure 3

See image above for figure legend.

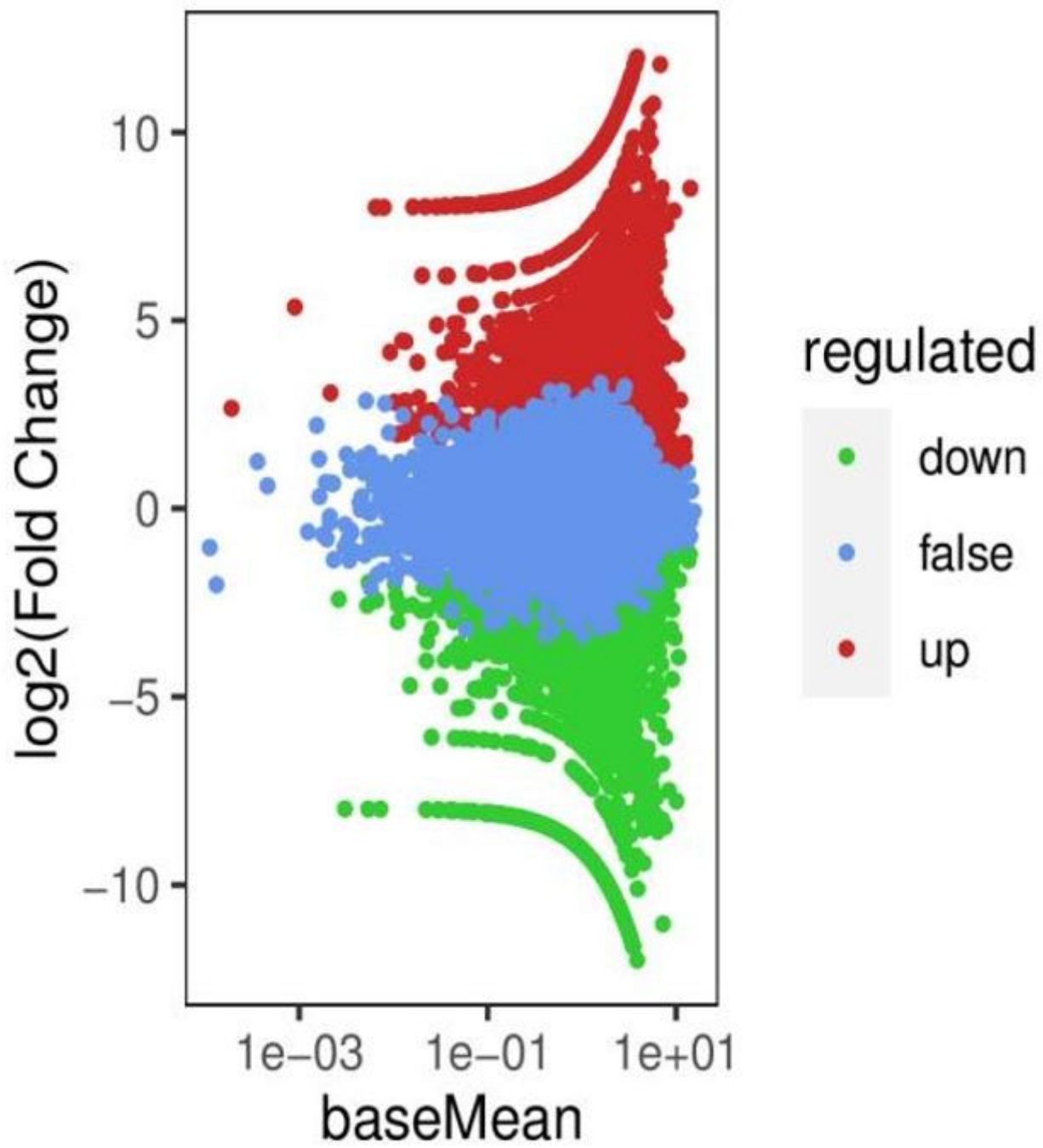


Figure 4

Volcano map of differential genes between Qssg and Lc fruits.

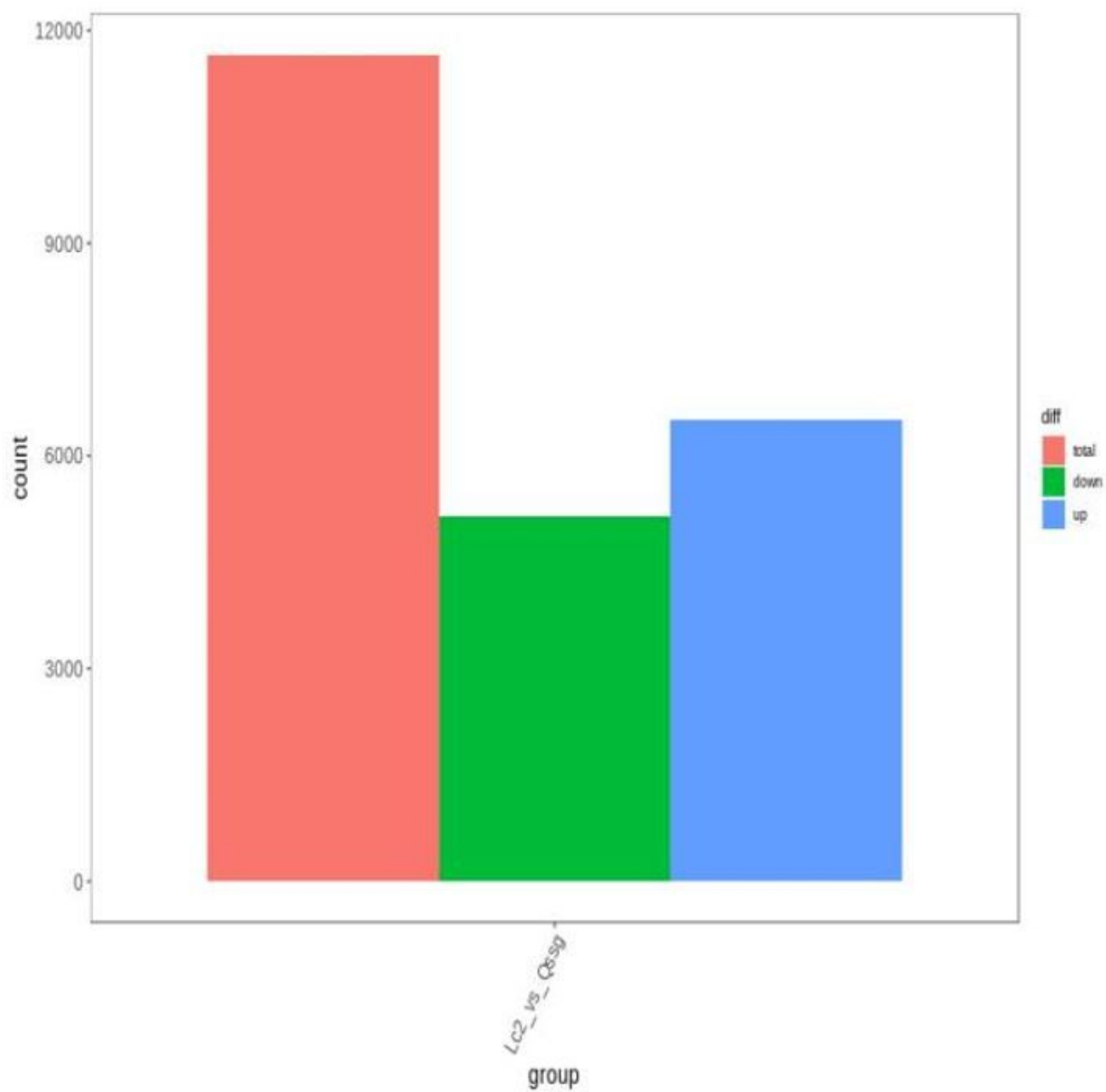


Figure 5

Columnar chart of the differential gene number in Qs5g and Lc fruits.

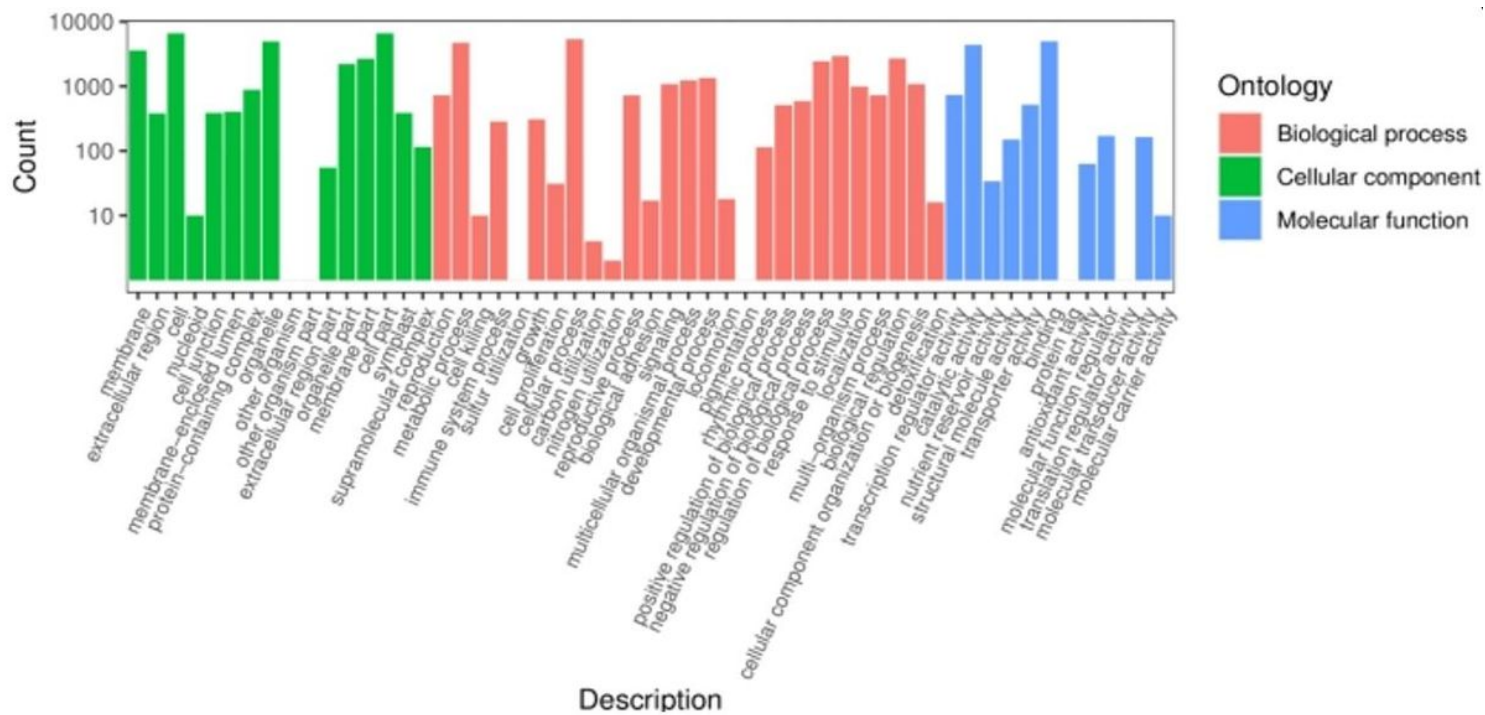


Figure 6

Classification of secondary entries of Qssg and Lc fruit difference genes.

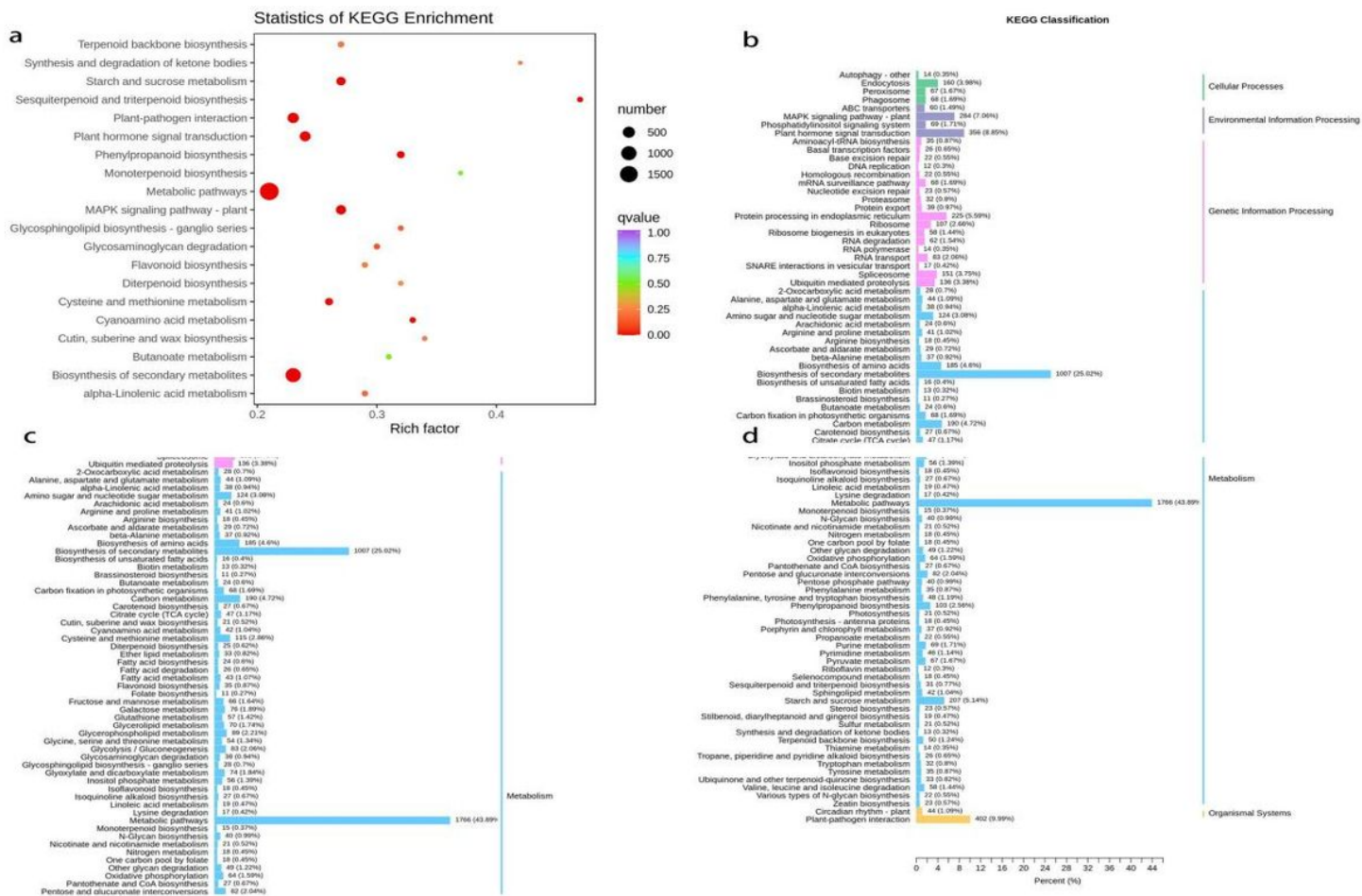


Fig. 7 Analysis results of differential gene expression in Qsg and Lc *Actinidia arguta* Sieb. Zucc.. **a** Scatter diagram of enrichment of differential gene KEGG in Qsg and Lc fruit. **(b-d)** Histogram of differential gene go enrichment.

Figure 7

See image above for figure legend.

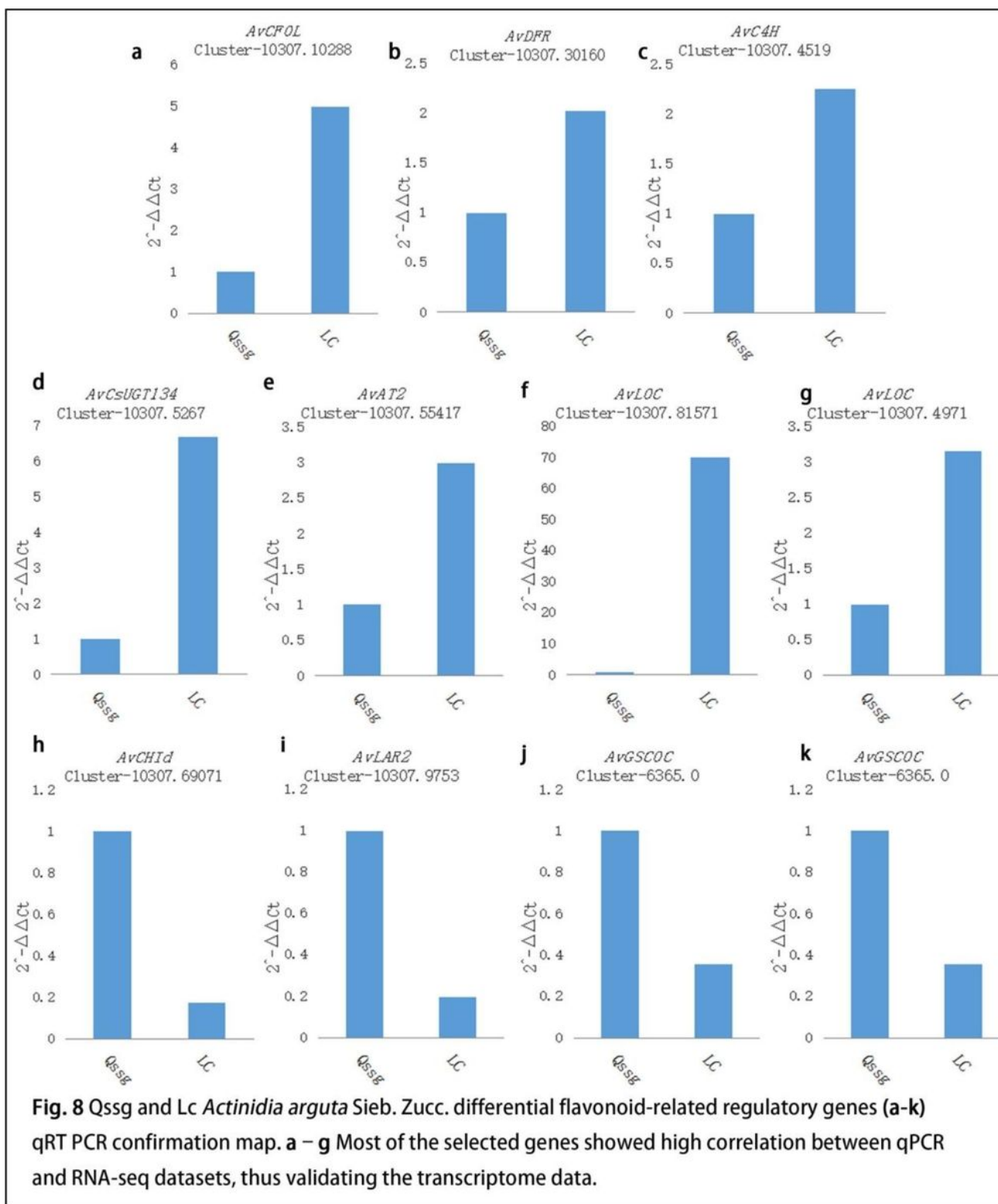


Figure 8

See image above for figure legend.

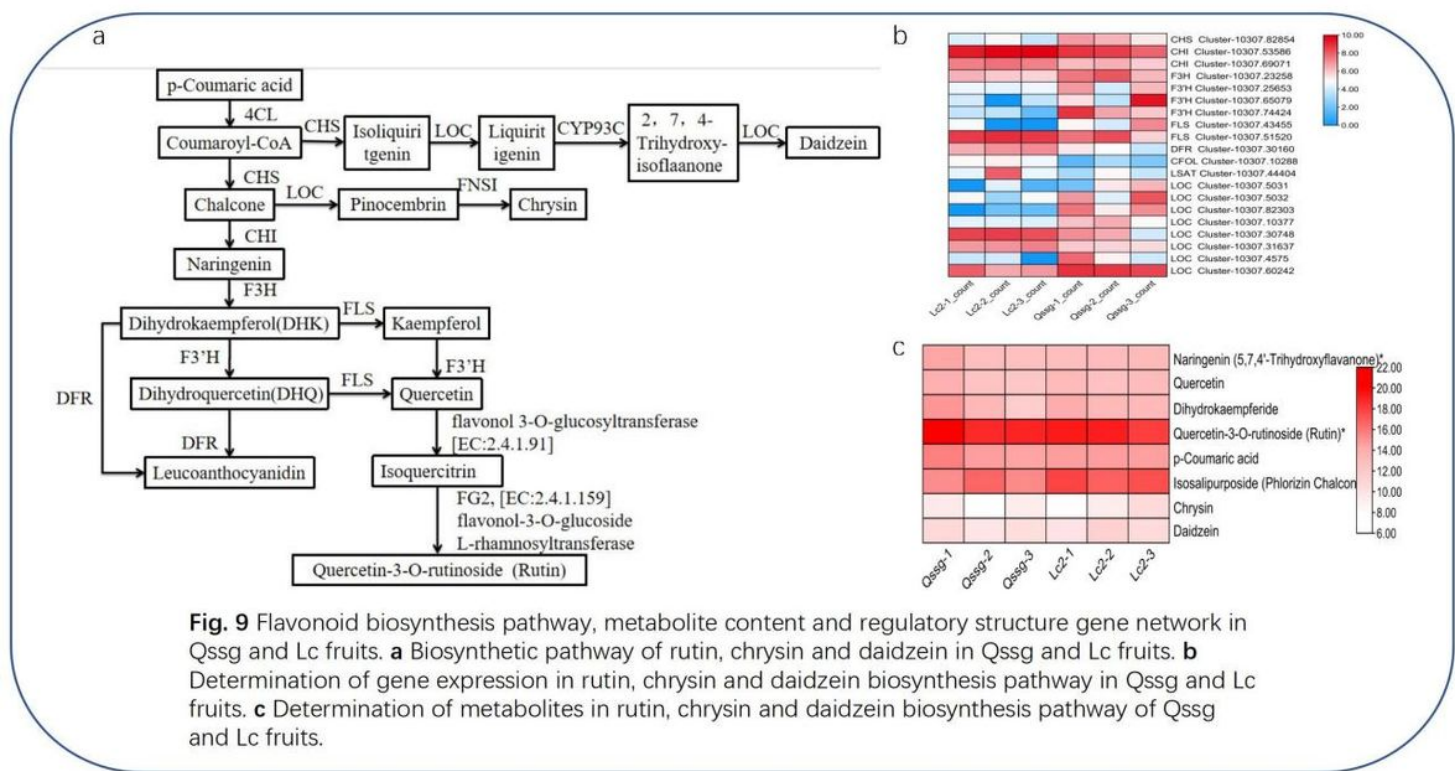


Figure 9

See image above for figure legend.