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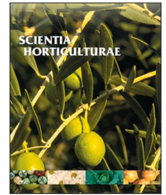

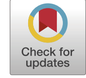

GC-MS/LC-MS and transcriptome analyses revealed the metabolisms of fatty acid and flavonoid in olive fruits (*Olea europaea* L.)

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ABSTRACT

Olive (*Olea europaea* L.) is an economical fruit tree for the usage of oil extraction and table olives. It is favored by consumers because of abundant unsaturated fatty acids and flavonoids, but little known about the genetic mechanisms. This study identifies the fruit traits of three olive cultivars 'Arbequina', 'Frantoio selection' and 'Nikitskii I', first planted in the conditions of acid soil and rainy summer and further elucidates the fatty acid and flavonoid biosynthesis mechanism by multi-omics analysis. 'Arbequina' and 'Frantoio selection' had medium flesh/pit ratios (3.94, 3.53) and oil contents (15.95%, 12.95%) and were suitable for oil extraction. 'Nikitskii I' had a big flesh/pit ratio (6.25) and medium oil content (13.13%) and could be used both for table olives and oil purpose. Totally, 37 fatty acid and 35 flavonoid compounds were detected by gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry technologies with the average Pearson correlation indexes of 0.985 and 0.971 among different cultivars, respectively. Transcriptome analysis identified 14,684 differentially expressed genes with 1008 common differential genes. Furthermore, enrichment analysis showed 15 and 8 pathways involved in fatty acid and flavonoid metabolism with 44 and 32 prior transcripts tested, respectively. Overall, among the three cultivars, 'Frantoio selection' and 'Nikitskii I' displayed a larger difference and they showed the high ratios of unsaturated fatty acids/fatty acids and oleic acid/fatty acids, respectively. While 'Arbequina' presented a big advantage in flavonoid compounds and expressions of related genes. The study provides the excellent materials and candidate genes for genetically improving of the quality of olive oil.

1. Introduction

Olive (*Olea europaea* L.) is a renowned woody oil tree and has been cultivated for around 6000 years in Mediterranean countries (Zohary and Hopf, 1994). It belongs to the Oleaceae family and is the only species that produces edible fruits within the *Olea* genus (Green, 2002; Green and Wickens, 1989). The olive fruits are rich in unsaturated fatty acids, polyphenols and various antioxidants. The two main products, olive oil and table olives, preserve the natural substances to the greatest extent (Kalua et al., 2007; Sebastiani and Busconi, 2017).

Olive oil contains 86.5% of unsaturated fatty acids, among which oleic acid comprises up to 83% (Li, 2010). Abundant monounsaturated fatty acids can balance the proportion of cholesterol in the blood and effectively improve the cardiovascular system (Sebastiani and Busconi, 2017). Antioxidant substances such as squalene, polyphenol compounds, and vitamin E are found in olives and are greatly beneficial to health (Kalua et al., 2007; Pérez-Jiménez et al., 2007). Researches showed that hydroxytyrosol, tyrosol, secoiridoids, lignans, peroxidation-resistant lipid, and oleic acid are the most important factors in the 'Mediterranean diet', which promotes lower incidences of cancer and cardiovascular disease (Owen et al., 2004). With the gradual attention to the effective ingredients, various compounds in olive fruits have also been extracted and fully exploited in the beauty and healthcare industries (Kalua et al., 2007; Owen et al., 2004; Pérez-Jiménez et al., 2007).

There are more than 2000 cultivars of olive trees in the world, including the 320 main cultispecies (Deng, 2018). OLEA, a public and comprehensive olive science database, contains accession information

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for more than 1000 olive cultivars and records their agronomical, biochemical, and molecular marker traits (Bartolini et al., 2005). Fruit characteristics vary by the stage of ripeness and growth environmental conditions, especially genetic diversity. Cluster analysis of 361 olive accessions showed that genetic information and geographic origin were closely related to genetic diversity (Belaj et al., 2012). The genetic classification of the different cultivars also appeared to be correlative with fruit size (Zhu et al., 2019). The utilization of transcriptomics and metabolomics has greatly accelerated our understanding of the metabolites and developmental processes of olive trees. Roche-454 massive parallel pyrosequencing of the pericarp and abscission zones in 'Picual' ripe fruit revealed differentially expressed genes (DEGs) related to auxin signaling, lignin, aromatic amino acid, isoprenoid, and the protein dephosphorylation biosynthetic pathway (Parra et al., 2013). Transcriptomic analysis of five developmental fruit stages for the cultivar 'Koroneiki' found active gene expression and metabolite accumulation related to tyrosol, hydroxytyrosol, and oleuropein biosynthesis (Mougiou et al., 2018). Rao et al. (2019) measured 12 polyphenols in five developmental stages of the cultivar 'Leccino' fruits and three aging degrees of leaves, and identified 122, 101 and 106 transcripts involved in flavonoid, oleuropein and hydroxytyrosol biosynthesis using full-length transcriptome sequencing. The olive is an ancient tree and has been favored for thousands of years, but the molecular studies have been relative slow. This imbalance requires more efforts to make full use of traditional and molecular breeding methods to improve the yield and quality of olive oil.

After long-term natural domestication and selection, olive has become a crop well adapted to the environmental conditions of Mediterranean Basin. Nevertheless, the increasing international demand for olive oil and table olives in the last two decades has led to expansion of olive industry worldwidely. There have been more than 40 countries that have introduced and cultivated olive trees today (Kaniewski et al., 2012). Due to the great environmental differences with the traditional growing area, the cultivars from Mediterranean basin generally have adaptability problems with different levels of acid soil and rainy environment. Field test so far shown that three cultivars 'Arbequina', 'Frantoio selection' and 'Nikitskii I' were first identified to be suitable well for growing in the environment (Niu et al., 2021). This study will further conduct the analysis of compositions and biosynthesis of fatty acid and flavonoid biosynthesis for the three cultivars, aiming to identify the specific metabolic component and putative genes for functional product development and genetic improvement.

2. Materials and methods

2.1. Field site and plant materials

Tests were conducted in Chun'an, Zhejiang Province, China, in the olive experimental field of the Zhejiang Academy of Agricultural Sciences (29°11′~30°02′N, 118°20′~119°20′W, 375 m asl.). The farm experiences a hot-rainy summer and cold-dry winter with the annual average temperature of 17 °C and rainfall of 1517 mm. The terrain consists of hills with red soil (pH= 4.98~5.33).

Three olive cultivars, 'Arbequina', 'Frantoio selection' and 'Nikitskii I', were planted with a 4 *m* × 5 m spacing. During the second year of the initial bearing stage, i.e. at 5 years of tree age, fruits with the Maturity index= 4 (Muzzalupo, 2012a) were collected for characteristic determination. A random selection of fruits was quickly frozen in liquid nitrogen and stored at −80 °C freezer for metabolome and transcriptome analysis.

2.2. Determination of morphological and agronomical characteristics

Fruits were selected at random for the following characteristic investigations: weight, polar length, cross-sectional width, shape index of single fruit and stone, flesh/pit ratio, and oil content. Three biological replicates were performed for each trait and each replicate included 10 fruits. Of them, shape index was determined by the ratio of polar length and cross-sectional width (Muzzalupo, 2012a). Olive oil was also extracted via the Soxhlet extraction method (Castro and Priego-Capote, 2010). The amount of fresh fruit was weighed appropriately (W_0) and powdered immediately. The powdered samples were transferred to the filter paper and baked at 60 °C for 24 h, and then at 105 °C to a constant weight (W_1). Another filter paper was used to seal the dried sample, which was then placed in Soxhlet extractor with anhydrous ether for 12 h. After the sample was baked to a constant weight (W_2), the oil content was recorded as $((W_1\text{-}W_2)/W_0) \times 100\%$.

2.3. Extraction of fatty acids and gas chromatography-mass spectrometry (GC-MS) procedure

One milliliter of Chloroform/methanol (2:1) and 100 mg of glass beads were added into each powdered fruit sample (100 mg). The mixture was shocked twice at 60 Hz for 1 min in a high-throughput tissue mill and then sonicated in an ultrasonic cleaner at room temperature for 30 min. After centrifugation, 800 μL of supernatant was collected and mixed with 2 mL of a 1% sulfuric acid methanol solution. Samples were esterified in a water bath at 80 °C for 30 min. One milliliter of alkane and 5 mL ddH_2O were added before centrifuging at 4 °C for 10 min. One hundred milligrams of anhydrous sodium sulfate (powder) was added to 700 μL of supernatant to remove excess water. Five hundred microliters of each sample was diluted 20 times and was then analyzed by GC-MS methodology (Liu et al., 2021). Six biological replicates were performed for each sample.

For the GC-MS procedure, chromatographic separations were performed on a HP-INNOWAX capillary column (30 *m* × 0.25 mm ID, 0.25 μm). The injection volume was 1 μL. The inlet temperature was 250 °C, ion source temperature was 230 °C, transfer line temperature was 250 °C, and quadrupole temperature was 150 °C. Temperature programs were as follows: initial temperature = 50 °C and hold for 3 min; rise to 220 °C at a speed of 10 °C/min and hold for 3 min; finally rise to 250 °C at a speed of 15 °C/min and hold for 10 min. The carrier gas was helium with flow rate 1.0 mL/min. MS conditions were as follows: electron bombardment ionization source; scan mode, single ion monitoring; electron energy, 70 eV. The ion pairs used for quantitative analysis were shown in Supplementary Table 1.

2.4. Extraction of flavonoids and liquid chromatography-mass spectrometry (LC-MS) procedure

Six hundred microliters of methanol were added to each powdered sample (100 mg). The mixture was sonicated in an ultrasonic cleaner at room temperature for 30 min. After centrifuging at 4 °C for 10 min, 300 μL of supernatant was collected and filtered through a 0.22 μm Millipore membrane. The filtrate was then analyzed by LC-MS methodology (Zhou et al., 2021). Six biological replicates were performed for each sample.

For the LC-MS procedure, chromatographic separations were performed on a Waters ACQUITY UPLC® BEH C18 column (2.1 × 100 mm, 1.7 μm). The injection volume was 5 μL, with a flow rate of 0.25 mL/min and the column temperature was 40 °C. Formic acid aqueous water and methanol were employed as Eluents A1 and B. Gradient elution conditions were as follows: 0–1 min, 10% B; 1–3 min, 10%–33% B; 3–10 min, 33% B; 10–15 min, 33%–50% B; 15–20 min, 50%–90% B; 20–21 min, 90% B; 21–22 min, 90%–10% B; 22–25 min, 10% B. The MS detection conditions were as follows: electrospray ionization source, negative ionization mode; ionization temperature, 500 °C; ionization voltage, −4500 V, collision gas, 6 psi; curtain gas, 30 psi, atomizing gas and auxiliary gas, 50 psi; scan mode, multiple reaction monitoring. The ion pairs used for quantitative analysis were shown in Supplementary Table 2.

2.5. RNA-sequencing and DEG enrichment

Total RNA was obtained from the fruits of three cultivars using the CTAB with three replicates. Gel electrophoresis and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) were used to check the quality of RNA and further quantify it. After concentrated and distributed by an Agilent2100 bioanalyzer, the library DNA was sequenced with an Illumina HiSeq 2500 system according to the manufacturer's instructions (HiSeq 2500 User Guide). The raw data had been submitted as PRJNA690674 in the National Center for Biotechnology Information (NCBI).

To explore the DEGs, Python software was used for mapping the reads to the olive reference genome *O. europaea* cv. Farga (Cruz et al., 2016) and expression levels for each gene were represented as fragments per kilobase of the exon model per million mapped reads (FPKM). DEGs were identified by Cuffdiff and were required to have a 2-fold change and Q value $\leq$ 0.01. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis were conducted using agirGO and KOBAS (Du et al., 2010). Additionally, 20 genes were selected to conduct the quantitative real-time PCR (qRT-PCR) in two random samples to conduct a Pearson correlation analysis between RNA-seq and qRT-PCR results. The gene *OeActin* (OE6A099235) was used as the endogenous control, and the primers of 20 genes were listed in Supplementary Table 3.

2.6. Statistical analysis

Statistical analyses were performed using SPSS Statistics 23 (IBM Corp. Armonk, New York, USA). All the parameters of different cultivars were displayed as mean values $\pm$ standard deviation. General Linear Model ANOVA analysis with Tukey's test was used to confirm the significant differences at a level of $P<0.05$.

3. Results

3.1. Morphological and agronomical traits of 'Arbequina', 'Frantoio selection' and 'Nikitskii I' fruits

Three olive cultivars, 'Arbequina', 'Frantoio selection' and 'Nikitskii I' were planted in acid soil and a rainy environment and the fruit characteristics were shown in Fig. 1 and Table 1. The fresh fruit weight, polar length, cross sectional width, and shape index were significantly different among cultivars. The fruit weight was highest in 'Nikitskii I' (4.18 g, $P<0.05$) and lowest in 'Arbequina' (1.79 g, $P<0.05$). These two cultivars also had the highest and lowest fruit polar length, cross sectional width, and shape index ('Nikitskii I', 26.81 mm, 18.53 mm, 1.45, and 'Arbequina', 15.48 mm, 13.01 mm, 1.19, $P<0.05$). The fruits of 'Nikitskii I' and 'Frantoio selection' displayed an elliptic shape, while 'Arbequina' had a spherical shape. The stone characteristics were not entirely consistent with the fruits. 'Frantoio selection' had the highest stone weight, polar length, and cross-sectional width (0.61 g, 17.42 mm, 8.53 mm), and 'Arbequina' had the lowest (0.36 g, 11.78 mm, 7.72 mm). 'Nikitskii I' and 'Frantoio selection' had stone shape indexes of 2.10 and 2.04, respectively, showing an elliptic shape, while 'Arbequina' had a stone shape index of 1.53, showing an ovoid shape. 'Nikitskii I' had the biggest flesh/pit ratio (6.25) and medium oil content (13.13%) and could be used both for table olives and olive oil, while 'Arbequina' and 'Frantoio selection' were more suitable for oil extraction.

3.2. Comparison of fatty acid and flavonoid compounds by GC-MS and LC-MS procedure

To compare the quality of different olive cultivars, 37 fatty acid and 35 flavonoid compounds were determined in 'Arbequina', 'Frantoio selection' and 'Nikitskii I' fruits with six biological replicates. The contents of 11 fatty acid compounds were too low to be detected in more than half of the samples including Butyric acid (C4:0), Caproic acid (C6:0), Caprylic acid (C8:0), Capric acid (C10:0), Undecanoic acid (C11:0), Lauric acid (C12:0), Tridecanoic acid (C13:0), Pentadecenoic acid (C15:1), Arachidonic acid (C20:4N6), cis-11,14,17-Eicosatrienoic acid (C20:3N3), and Timnodonic acid (C20:5N3). As the result, the fatty acid compositions of the three cultivars were similar, with a Pearson correlation index of 0.996 ('Arbequina' vs 'Nikitskii I'), 0.988 ('Arbequina' vs 'Frantoio selection'), and 0.970 ('Frantoio selection' vs 'Nikitskii I') (Table 2). The unsaturated fatty acids (UFAs) accounted for 74.82%, 77.03%, and 75.10% of total fatty acids (FAs) in 'Arbequina', 'Frantoio selection' and 'Nikitskii I' fruits, respectively. Oleic acid (C18:1), Linoleic acid (C18:2), and Palmitic acid (C16:0) were the three most abundant fatty acids, with the average contents of 20,510.25 (19,531.29~21,053.13), 15,094.92 (12,143.20~18,326.08), and 10,401.68 (9820.53~10,864.55) μg/g fresh weight (FW), respectively. 'Arbequina' recorded the highest contents of C18:1 (21,053.13 μg/g FW) and C16:0 (10,864.55 μg/g FW), while 'Frantoio selection' had the richest content of C18:2 (18,326.08 μg/g FW) in its fruits ($P<0.05$). In addition to the three fatty acids, Palmitoleic acid (C16:1), Stearic acid (C18:0) and Linolenic acid (C18:3N3) were also higher with contents > 1000.00 μg/g FW in individual cultivar. In the remaining fatty acids, the contents of Heptadecenoic acid (C17:1) in 'Arbequina' and 'Nikitskii I' were up to 4.23 and 3.29 times that of 'Frantoio selection' ($P<0.05$), and the content of Heptadecanoic acid (C17:0) in 'Arbequina' was 3.16, 1.66 times that of 'Nikitskii I' and 'Frantoio selection' ($P<0.05$). These two compounds were intermediates in the synthesis of polyunsaturated fatty acids, which would be largely related to the genetic differences and needs to be further explored.

Among 35 flavonoid compounds, the contents of 13 were too low to be detected in more than half samples including Baicalin, Biochanin A,

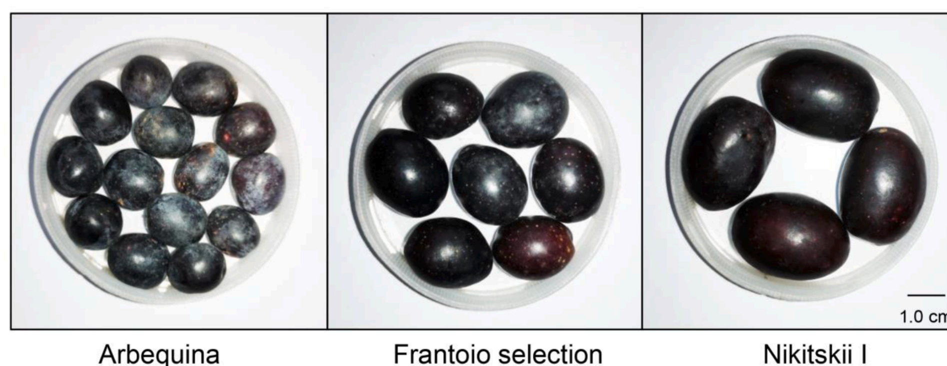

Fig. 1. Fruit traits of three olive cultivars 'Arbequina', 'Frantoio selection' and 'Nikitskii I'. Three olive cultivars were planted in acid soil and rainy environment for five years and the fruits with the Maturity index= 4 were sampled. Bar= 1.0 cm.

Table 1
Morphological and agronomical traits of ‘Arbequina’, ‘Frantoio selection’ and ‘Nikitskii I’ fruits.

Cultivars	Fruits				Stones				Flesh/pit ratio	Oil content/%
	Weight/g	Polar length/mm	Cross sectional width/mm	Shape index	Weight/g	Polar length/mm	Cross sectional width/mm	Shape index		
Arbequina	1.79 ±0.38 c	15.48±0.82 c	13.01±0.79 c	1.19 ±0.03 c	0.36 ±0.08 b	11.78±0.81 c	7.72±0.38 b	1.53 ±0.10 b	3.94	15.94 ±1.61 a
Frantoio selection	2.78 ±0.40 b	19.11±1.62 b	14.09±0.81 b	1.36 ±0.07 b	0.61 ±0.13 a	17.42±1.17 a	8.53±0.60 a	2.04 ±0.10 a	3.53	12.95 ±1.81 a
Nikitskii I	4.18 ±1.09 a	26.81±2.43 a	18.53±1.15 a	1.45 ±0.06 a	0.58 ±0.06 a	16.52±1.12 b	7.88±0.33 b	2.10 ±0.14 a	6.25	13.13 ±1.70 a

All the traits of different cultivars were displayed as mean values ± standard deviation. Different letters indicated the significant differences at a level of $P<0.05$ by Tukey’s test.

Table 2
Individual fatty acid composition in three olive cultivars.

Composition (μg/g FW)	Arbequina	Frantoio selection	Nikitskii I	Composition (μg/g FW)	Arbequina	Frantoio selection	Nikitskii I
C18:1	21,053.13±1531.73 a	19,531.29±1535.40 a	20,946.33±335.95 a	C14:0	30.98±0.98 a	22.07±0.68 c	28.85±0.54 b
C18:2	14,815.49±754.92 b	18,326.08±1001.70 a	12,143.20±171.27 c	C24:1	18.81±3.45 c	39.43±2.70 a	32.31±3.58 b
C16:0	10,864.55±555.08 a	9820.53±522.00 b	10,519.98±248.40 ab	C21:0	18.00±1.91 a	16.14±1.77 a	15.11±1.19 a
C16:1	3301.10±139.51 b	2169.97±58.47 c	3533.76±30.57 a	C15:0	15.93±1.10 a	8.47±0.39 b	14.47±0.27 a
C18:0	1954.64±102.45 a	1904.16±59.03 a	1646.12±32.89 a	C18:3N6	15.76±0.60 b	20.42±0.54 a	13.10±0.36 b
C18:3N3	888.07±35.47 c	1351.78±63.89 a	1205.29±15.16 b	C22:1N9	13.40±2.53 b	15.62±2.07 ab	17.11±2.56 a
C20:0	436.01±20.96 a	397.29±16.97 b	297.61±3.85 c	C22:6N3	12.20±1.72 c	31.76±1.23 a	22.07±0.90 b
C20:1	328.39±11.19 a	312.62±10.51 a	312.97±4.44 a	C20:3N6	7.63±0.92 ab	6.30±0.52 b	7.91±0.76 a
C17:1	298.50±14.26 a	70.50±2.08 c	231.77±4.62 b	C22:2	6.74±3.77 a	6.70±3.58 a	7.10±1.20 a
C22:0	145.50±5.11 a	152.78±4.98 a	81.76±1.78 b	C20:2	6.50±0.91 a	8.80±0.72 a	6.60±0.67 a
C17:0	127.41±6.56 a	40.31±1.34 c	76.58±1.27 b	C14:1	6.38±1.01 b	9.87±0.64 a	5.40±2.79 b
C24:0	94.60±8.20 a	93.18±6.49 a	58.69±3.76 b	UFAs/FAs	74.82±0.26% b	77.03±0.31% a	75.10±0.21% b
C23:0	31.05±4.18 a	32.66±5.17 a	25.36±2.24 b	C18:1/FAs	38.61±0.72% b	35.88±0.79% c	40.88±0.30% a

C4:0: Butyric acid; C6:0: Caproic acid; C8:0: Caprylic acid; C10:0: Capric acid; C11:0: Undecanoic acid; C12:0: Lauric acid; C13:0: Tridecanoic acid; C14:0: Myristic acid; C14:1: Myristoleic acid; C15:0: Pentadecanoic acid; C15:1: Pentadecenoic acid; C16:0: Palmitic acid; C16:1: Palmitoleic acid; C17:0: Heptadecanoic acid; C17:1: Heptadecenoic acid; C18:0: Stearic acid; C18:1: Oleic acid; C18:2: Linoleic acid; C18:3N6: γ-Linolenic acid; C18:3N3: Linolenic acid; C20:0: Arachidic acid; C20:1: Eicosenoic acid; C20:2: Eicosadienoic acid; C21:0: Henicosanoic acid; C20:3N6: cis-8:11:14-Eicosatrienoic acid; C20:3N3: cis-11:14:17-Eicosatrienoic acid; C20:4N6: Arachidonic acid; C20:5N3: Timnodonic acid; C22:0: Behenic acid; C22:1N9: Erucic acid; C22:2: Docosadienoic acid; C22:6N3: Docosahexaenoic acid; C23:0: Tricosanoic acid; C24:0: Lignoceric acid; C24:1: Nervonic acid; FW: Fresh weight.The concentrations of C18:1 and Elaidic acid (C18:1T), C18:2 and Linoelaidic acid (C18:2TT) were merged as C18:1 and C18:2, respectively, because of the same isomers forms. All the traits of different cultivars were displayed as mean values ± standard deviation. Different letters indicated the significant differences at a level of $P<0.05$ by Tukey’s test.

Catechin, Daidzein, Dihydromyricetin, Fisetin, Formononetin, Genistein, Glycitein, Kaempferide, L-Epicatechin, Liquiritigenin, and Myricetin. The three cultivars demonstrated high similarity of the flavonoid compounds with a Pearson correlation index of 0.978 (‘Arbequina’ vs ‘Frantoio selection’), 0.986 (‘Arbequina’ vs ‘Nikitskii I’) and 0.950 (‘Frantoio selection’ vs ‘Nikitskii I’), respectively (Table 3). The total contents of flavonoid compounds were 801.87 (‘Arbequina’), 365.72 (‘Frantoio selection’) and 341.60 μg/g FW (‘Nikitskii I’), respectively, showing the obvious superiority of the cultivar ‘Arbequina’. The top four abundant flavonoid compounds were Luteolin, Rutin, Cynaroside and Kaempferol with the percentages in total flavonoid compounds of 93.52% (‘Arbequina’), 86.45% (‘Frantoio selection’) and 89.31% (‘Nikitskii I’), respectively. Interestingly, ‘Arbequina’ had the highest contents of all these four flavonoids, which was more than twice that of

Table 3
Individual flavonoid composition in three olive cultivars.

Composition (μg/g FW)	Arbequina	Frantoio selection	Nikitskii I	Composition (μg/g FW)	Arbequina	Frantoio selection	Nikitskii I
Luteolin	373.03±38.38 a	181.57±29.13 b	139.96±16.47 c	Naringenin	2.09±0.15 a	0.75±0.12 b	0.88±0.11 b
Rutin	243.33±22.5 a	81.43±10.86 c	121.2 ± 16.16 b	Naringin	1.51±0.24 a	0.48±0.03 c	1.08±0.21 b
Cynaroside	104.69±13.77 a	38.77±5.1 b	33.52±5.02 b	Diosmin	0.98±0.15 a	0.19±0.05 b	0.24±0.04 b
Kaempferol	28.82±2.71 a	14.39±2.33 b	10.41±1.55 c	Vitexin	0.36±0.07 a	0.17±0.02 b	0.13±0.02 b
Glycitin	13.56±4.5 a	3.8 ± 1.52 b	3.27±1.17 b	Isovitexin	0.23±0.03 a	0.08±0.02 b	0.11±0.04 b
Apigenin	9.5 ± 0.71 a	9.11±1.3 a	5.12±0.49 b	Quercitrin	0.2 ± 0.17 c	22.91±2.96 a	10.42±1.57 b
Quercetin	7.33±0.72 a	5.03±1.32 b	5.83±0.99 b	Silybin	0.03±0.01 a	0.05±0.03 a	0.04±0.01 a
Quercetin 3-glucoside	5.82±0.53 a	2.37±0.24 b	5.91±0.74 a	Genistin	0.02±0 a	0.02±0.01 a	0.02±0.01 a
Daidzin	3.68±0.48 a	2.74±0.34 b	1.7 ± 0.16 c	Icariin	0.02±0.01 a	0.02±0.01 a	0.02±0.01 a
Dihydroquercetin	3.54±0.29 a	0.7 ± 0.09 b	0.9 ± 0.11 b	Puerarin	0.01±0.01 a	0.02±0.01 ab	0.01±0 b
Astragalin	3.13±0.41 a	1.1 ± 0.09 b	0.83±0.15 b	Chrysin	0.01±0 a	0.02±0.01 a	0.01±0.01 a

FW: Fresh weight. All the traits of different cultivars were displayed as mean values ± standard deviation. Different letters indicated the significant differences at a level of $P<0.05$ by Tukey’s test.

other cultivars. While except for Rutin, 'Nikitskii I' had the lowest accumulation of Luteolin, Cynaroside, and Kaempferol. Furthermore, the contents of Glycitin and Dihydroquercetin in 'Arbequina' were up to 3.56 and 5.03 times that of 'Frantoio selection' ($P<0.05$), and 4.15 and 3.91 times that of 'Nikitskii I' ($P<0.05$). On the contrary, the content of Quercitrin in 'Arbequina' was 0.20 μg/g FW, which was only 0.01 and 0.02 times of 'Frantoio selection' and 'Nikitskii I' ($P<0.05$). Glycitin and Dihydroquercetin had bacteriostatic effects and can kill the harmful bacteria in the intestine (Lu et al., 2006). Quercitrin and Myricetin were found to participate in strong anticancer activities by the inhibition of thioredoxin reductase (Lu et al., 2006). Cultivars with high content of these special compounds could be used for making specific value-added products.

3.3. Identification and enrichment of the DEGs

Transcriptome analysis of three olive cultivars with three biological replicates was further conducted for gene identification. In total, an average of 45.45 million clean reads was captured from the tested samples. After alignment to the olive reference sequences, *O. europaea* cv. Farga (Cruz et al., 2016), 42.53 million reads were obtained with an average mapping rate of 93.57% (Supplementary Table 4). The FPKM was employed to calculate the expression level of each gene, and the Pearson correlation coefficient was 89.88% with the results of qRT-PCR (Supplementary Fig. 1). In summary, the transcript expressions of 'Arbequina' and 'Nikitskii I' showed higher similarity with the correlation coefficient of 0.86, differing from the cultivar 'Frantoio selection' (Supplementary Fig. 2), indicating the diversity of 'Frantoio selection' from the other two cultivars.

With the cutoffs of Q value $\leq$ 0.01 and log2|fold change| $\geq$ 1, DEGs were identified between cultivars with 3583/3832, 4479/4698, and 4523/4067 DEGs (up/down) in 'Frantoio selection' vs 'Arbequina', 'Frantoio selection' vs 'Nikitskii I' and 'Nikitskii I' vs 'Arbequina', respectively (Fig. 2A). In total, 14,684 DEGs were found and 1008 genes were common differential genes in all three cultivars (Fig. 2B, 2C). Of them, five DEGs (OE6A043283, OE6A081536, OE6A107311, OE6A113898, and OE6A040828) were differentially expressed with a log2|fold change| > 10 (Fig. 2D). OE6A043283, encoding a nicotianamine synthase gene, serves as a sensor for physiological iron and is involved in the transport of iron (Inoue et al., 2003). OE6A081536 is involved in the detoxification of xenobiotics and used in cancer therapeutics (Chen et al., 2019). OE6A107311, an Sphase kinase-associated protein 1, plays an important role in lipid catabolic process. OE6A113898 (a pectin acetylesterase) and OE6A040828 (V-type *H*+-transporting ATPase subunit d2) seem related to the disease resistance (Kong et al., 2017). Due to the lack of research in gene function, the roles of these genes in olive trees have not been reported yet.

All the DEGs were mapped to the GO database using agirGO (Du et al., 2010) and the top 30 enrichment items are shown in Fig. 3. For 'Frantoio selection' vs 'Arbequina', 'Frantoio selection' vs 'Nikitskii I' and 'Nikitskii I' vs 'Arbequina', the most significantly enriched GO terms were "Structural constituent of ribosome (GO:0,003,735)," "Integral component of membrane (GO:0,016,021)," and "Oxidation reduction process (GO:0,055,114)," respectively. Three GO terms, "Transporter activity (GO:0,005,215)," "Integral component of membrane (GO:0,016,021)," and "Oxidation-reduction process (GO:0,055,114)" were coenriched (Fig. 3, Supplementary Table 5). KEGG analysis displayed the differential pathway more clearly. The top 30 pathways between each set of cultivars produced 54 pathways (Supplementary Table 6). Among them, 10 common pathways were enriched including "Tyrosine metabolism (ko00350)," "Phenylalanine/tyrosine/tryptophan biosynthesis (ko00400)," "β-Alanine metabolism (ko00410)," "Limonene and pinene degradation (ko00903)," "Phenylpropanoid biosynthesis (ko00940)," "Anthocyanin biosynthesis (ko00942)," "Stilbenoid/diarylheptanoid/gingerol biosynthesis (ko00945)," "Metabolic pathways (ko01100)," "Biosynthesis of secondary metabolites (ko01110)," and "Biosynthesis of amino acids (ko01230)," implying a differentiated physiological process occurring in the three cultivars, such as the

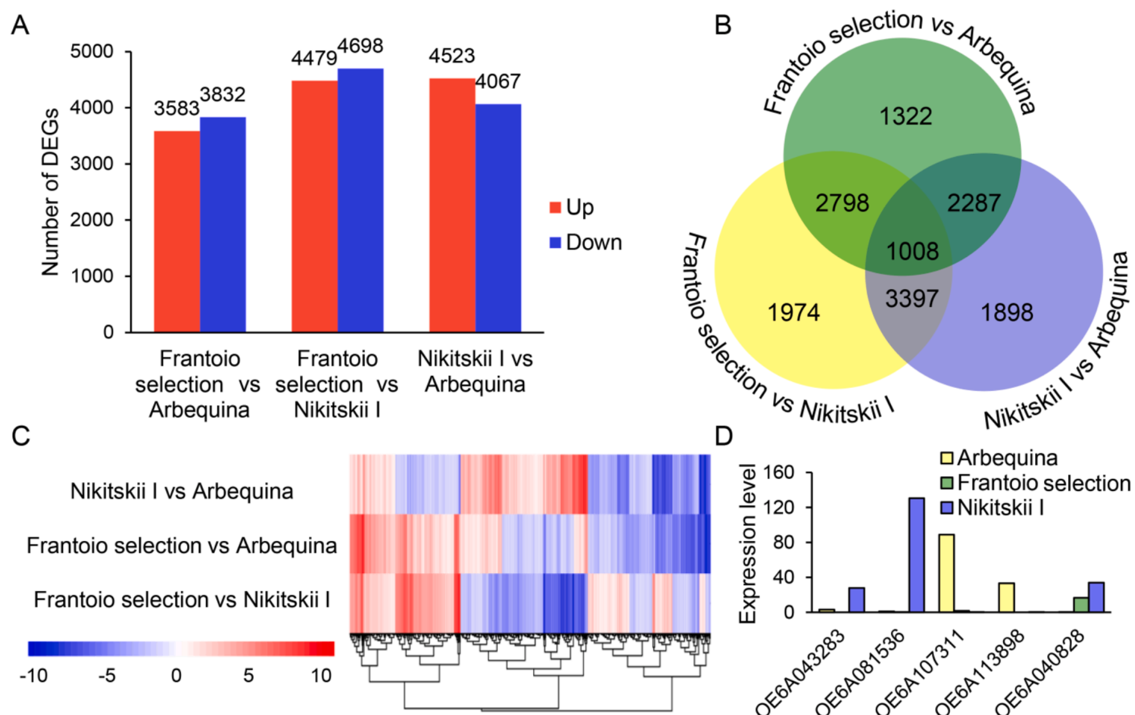

Fig. 2. Analysis of global differentially expressed genes (DEGs). (A/B) Statistics and Venn diagram of DEGs among the three cultivars. The expression level of each gene was represented as fragments per kilobase of exon model per million mapped reads (FPKM). DEGs were identified by Cuffdiff and were required to have a 2-fold change with a false discovery rate (FDR) <0.05. (C) Heat map of all DEGs. (D) Expression levels of five DEGs with the $\log_2$|fold change| > 10 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

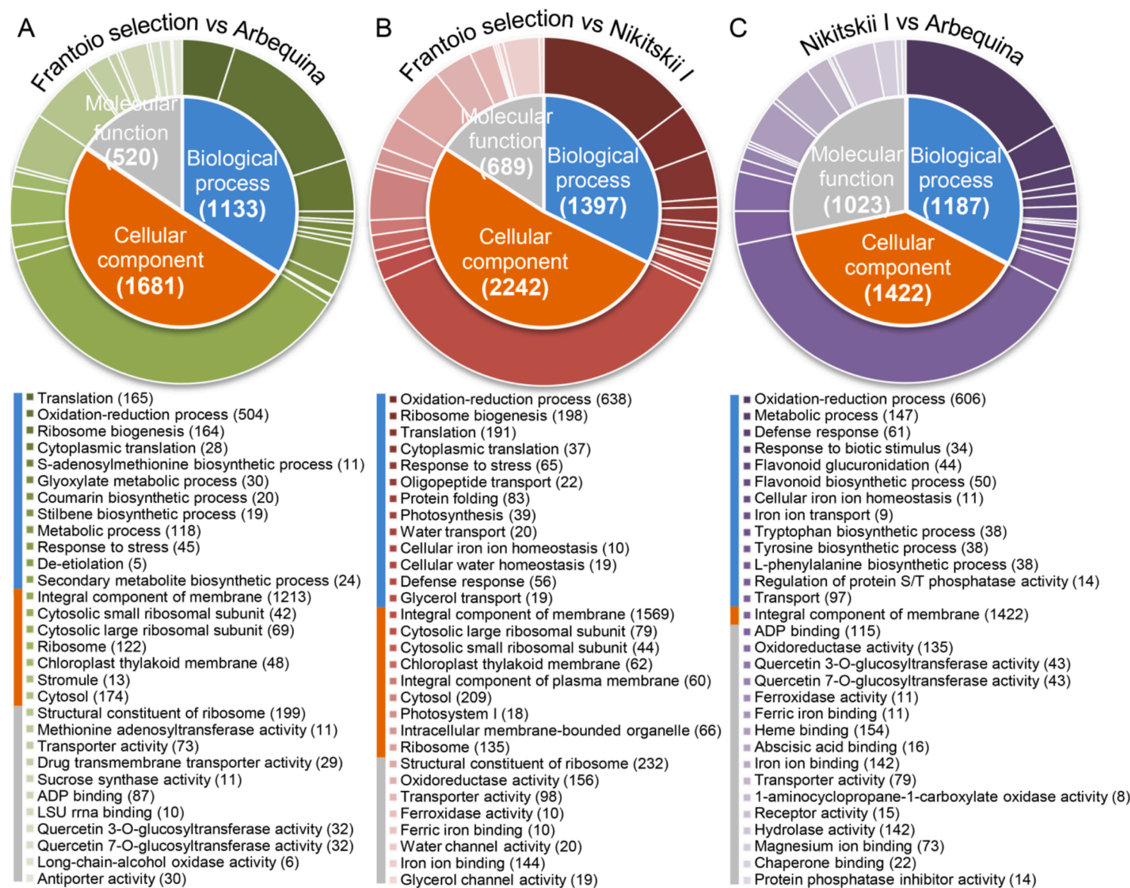

Fig. 3. Gene ontology (GO) category and genes numbers of differentially expressed genes (DEGs). The top 30 GO terms of DEGs ranked by Q values displayed as different colors. The inner and outer loops indicated the first and second GO categories (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

biosynthesis of essential amino acids, terpenoids, and phenols.

3.4. DEGs involved in fatty acid metabolism

The synthesis of fatty acids includes two processes: the initial synthesis and desaturation, which are mainly carried out in chloroplasts and endoplasmic reticulum, respectively (Sasaki and Nagano, 2004). Investigation of the KEGG pathways related to fatty acid biosynthesis showed that 15 pathways were detected among different cultivars including "ko00073 (Cutin, suberine and wax biosynthesis)," "ko00590 (Arachidonic acid metabolism)," "ko00062 (Fatty acid elongation)," "ko00071 (Fatty acid degradation)," "ko00592 (alpha-Linolenic acid metabolism), " "ko00072 (Synthesis and degradation of ketone bodies)," "ko00565 (Ether lipid metabolism)," "ko00561 (Glycerolipid metabolism)," "ko00591 (Linoleic acid metabolism)," "ko01040 (Biosynthesis of unsaturated fatty acids)," "ko00061 (Fatty acid biosynthesis)," "ko00600 (Sphingolipid metabolism)," "ko00100 (Steroid biosynthesis)," "ko00564 (Glycerophospholipid metabolism)," and "ko01212 (Fatty acid metabolism)" (Fig. 4A).

Acetyl-CoA carboxylase (ACCase) is a rate-determining point, which can convert acetyl-CoA into malonyl-CoA (Sasaki and Nagano, 2004). After catalysis by fatty acid synthesis, such as 3-ketoacyl-ACP synthase I (KAS I), KAS II, and KAS III, 16:0-ACP and 18:0-ACP are synthesized, and the latter converts into 18:1-ACP by Stearyl-ACP desaturase (SAD) catalysis (Slabas and Fawcett, 1992). Then, Acyl-ACP thioesterase (FAT), Long-chain acyl-CoA synthetase (LACS) and Lysophosphatidylcholine acyltransferase (LPCAT) are responsible for the formation of 18:1-PC and 18:1-PC is further desaturated by Fatty acid desaturase 2 (FAD 2) and FAD 3 (Du et al., 2019; Wang et al., 2015). Among the 10 key proteins related to fatty acid biosynthesis, 44 transcripts were differentially expressed between every two cultivars, including two ACCase (OE6A055531, OE6A095422), five KAS I (OE6A015649, OE6A118469, OE6A034683, OE6A079794, OE6A040462), two KAS III (OE6A067994, OE6A054505), four KAS II (OE6A120045, OE6A090667, OE6A089647, OE6A059052), five SAD (OE6A020845, OE6A048475, OE6A118450, OE6A012975, OE6A089828), eight FAT (OE6A065374, OE6A002888, OE6A017762, OE6A005678, OE6A007472, OE6A106158, OE6A047453, OE6A031168), ten LACS (OE6A055102, OE6A070068, OE6A012372, OE6A007296, OE6A034515, OE6A102185, OE6A097852, OE6A095994, OE6A093175, OE6A009852), one LPCAT (OE6A071537), five FAD2 (OE6A098403, OE6A051290, OE6A085290, OE6A011870, OE6A069627), and two FAD3 (OE6A075849, OE6A086562) (Fig. 4B, Supplementary Table 7). The total expression levels of the 10 proteins were further calculated in each individual cultivar (Fig. 4B). For 'Arbequina', KAS I and KAS III displayed higher expression levels than in 'Frantoio selection' and 'Nikitskii I', implying the active biosynthesis metabolisms of C16:0 occurred in 'Arbequina', which was consistent with the determination of metabolites (Table 2). For 'Nikitskii I', FAD3 had the highest expression levels; that is, more C18:2 was desaturated and transformed into C18:3, which might be the direct cause of the final content of C18:2 in 'Nikitskii I'. Except for KAS I, KAS III, and FAD3, the total expression levels of other proteins had the highest levels in 'Frantoio selection'. Among these enzymes, SAD, FAD2 and FAD3 are critical to affect the ratio of FAs and UFAs. In previous studies, most FAD2 and FAD3 proteins displayed lower expression levels in olive during fruit ripening and could be negligible (Hernández et al., 2015; Contreras et al., 2020). Here, all the five SAD genes had a higher expression with an average FPKM between

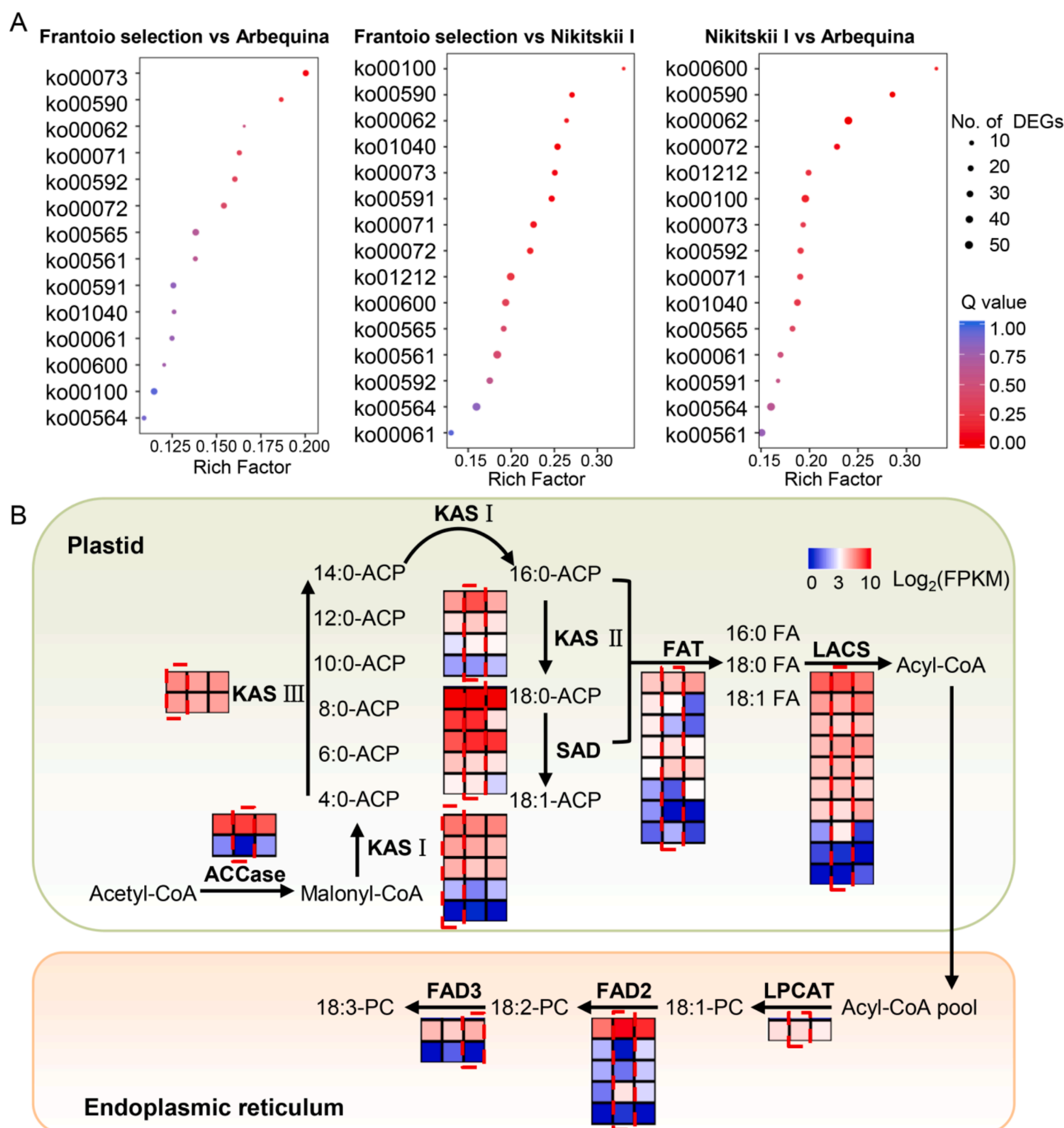

Fig. 4. Pathways and expression levels of differentially expressed genes (DEGs) related to fatty acid metabolism. (A) Kyoto encyclopedia of genes and genomes (KEGG) pathways related to fatty acid metabolism. The X-axis and Y-axis represent rich factors and different pathways. ko00073: Cutin, suberine, and wax biosynthesis; ko00590: Arachidonic acid metabolism; ko00062: Fatty acid elongation; ko00071: Fatty acid degradation; ko00592: alpha-Linolenic acid metabolism; ko00072: Synthesis and degradation of ketone bodies; ko00565: Ether lipid metabolism; ko00561: Glycerolipid metabolism; ko00591: Linoleic acid metabolism; ko01040: Biosynthesis of unsaturated fatty acids; ko00061: Fatty acid biosynthesis; ko00600: Sphingolipid metabolism; ko00100: Steroid biosynthesis; ko00564: Glycerophospholipid metabolism; ko01212: Fatty acid metabolism. (B) Molecular regulation and DEGs of fatty acid biosynthesis. The three cultivars were 'Arbequina', 'Frantoio selection' and 'Nikitskii I', and expression levels of DEGs were recorded as Log_2(FPKM). All transcripts of each type of protein were calculated in individual cultivars, and the cultivar with the highest transcripts was marked with a red dotted line. ACCase: Acetyl-CoA carboxylase; KAS: 3-ketoacyl-ACP synthase; SAD: Stearyl-ACP desaturase, FAT: Acyl-ACP thioesterase; LACS: Long-chain acyl-CoA synthetase; LPCAT: Lysophosphatidylcholine acyltransferase; FAD: Fatty acid desaturase (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

9.4 and 1016.5, while only one FAD2 (OE6A098403) and one FAD3 (OE6A075849) had an average FPKM > 10.0. Once again, this proved the superiority of oleic acid synthesis for olive tree. Furthermore, in addition to determining the content of oil compositions, fatty acids are an important supply for energy sources and resistance to low temperature stress, as well as act as precursors for the formation of aromatic substances (Miquel et al., 1993; Zhang et al., 2009). Therefore, mutations of these enzymes would also directly affect a variety of metabolic pathways of plants, which requires further exploration.

3.5. DEGs involved in flavonoid metabolism

Flavonoids were characterized into six categories including Flavones, Flavonols, Isoflavones, Flavanones, Flavanols, and Anthocyanidins and were synthesized through the phenylpropanoid pathway (Hollman and Arts, 2000; Chen et al., 2020). KEGG analysis showed that 8 pathways involved flavonoid biosynthesis were enriched including "ko00943 (Isoflavonoid biosynthesis)," "ko00360 (Phenylalanine metabolism)," "ko00942 (Anthocyanin biosynthesis)," "ko00944 (Flavone and flavonol biosynthesis)," "ko00941 (Flavonoid biosynthesis)," "ko00400 (Phenylalanine, tyrosine and tryptophan biosynthesis)," "ko00770 (Pantothenate and CoA biosynthesis)", "ko00940

(Phenylpropanoid biosynthesis)," (Fig. 5A).

Phenylalanine ammonialyase (PAL) is the first enzyme of the phenylpropanoid metabolic pathway and further converts Phenylalanine into ρ-coumaroyl-CoA with the activation of 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL), which is used as the substrate for flavonoid synthesis34. Modified by Chalcone synthase (CHS) and Chalcone isomerase (CHI), ρ-coumaroyl-CoA converts into Naringenin, which is an important intermediate and is catalyzed by different enzymes to form various flavonoids (Hollman and Arts, 2000; Chen et al., 2020; Ferrer et al., 2008; Long et al., 2019). Among the main proteins related to flavonoid metabolism, 32 DEGs were differentially expressed between each set of cultivars including six PAL (OE6A048764, OE6A099774, OE6A095147, OE6A028766, OE6A049944, OE6A003487), two C4H (OE6A098796, OE6A108606), seven 4CL (OE6A039066, OE6A083915, OE6A018525, OE6A051152, OE6A055071, OE6A023962, OE6A022766,), one CHS (OE6A012483), two CHI (OE6A004801, OE6A066812), seven Flavonoid 3′-hydroxylase (F3′H) (OE6A105218, OE6A068581, OE6A053412, OE6A052367, OE6A034639, OE6A056383, OE6A035864), one Flavone synthase (FNS) (OE6A081156), two Flavanone 3-hydroxylase (F3H) (OE6A028296, OE6A003772), and four Flavonol synthase (FLS) (OE6A040780, OE6A055813, OE6A082812, OE6A109123) (Fig. 5B, Supplementary Table 7). Excluding 4CL, F3H, and FNS, all transcripts of the other genes had the highest levels in 'Arbequina', which was in agreement with the active flavonoid accumulation in 'Arbequina' (Table 3). Previous studies show that besides flavonoid metabolism, PAL, C4H, and 4CL are also

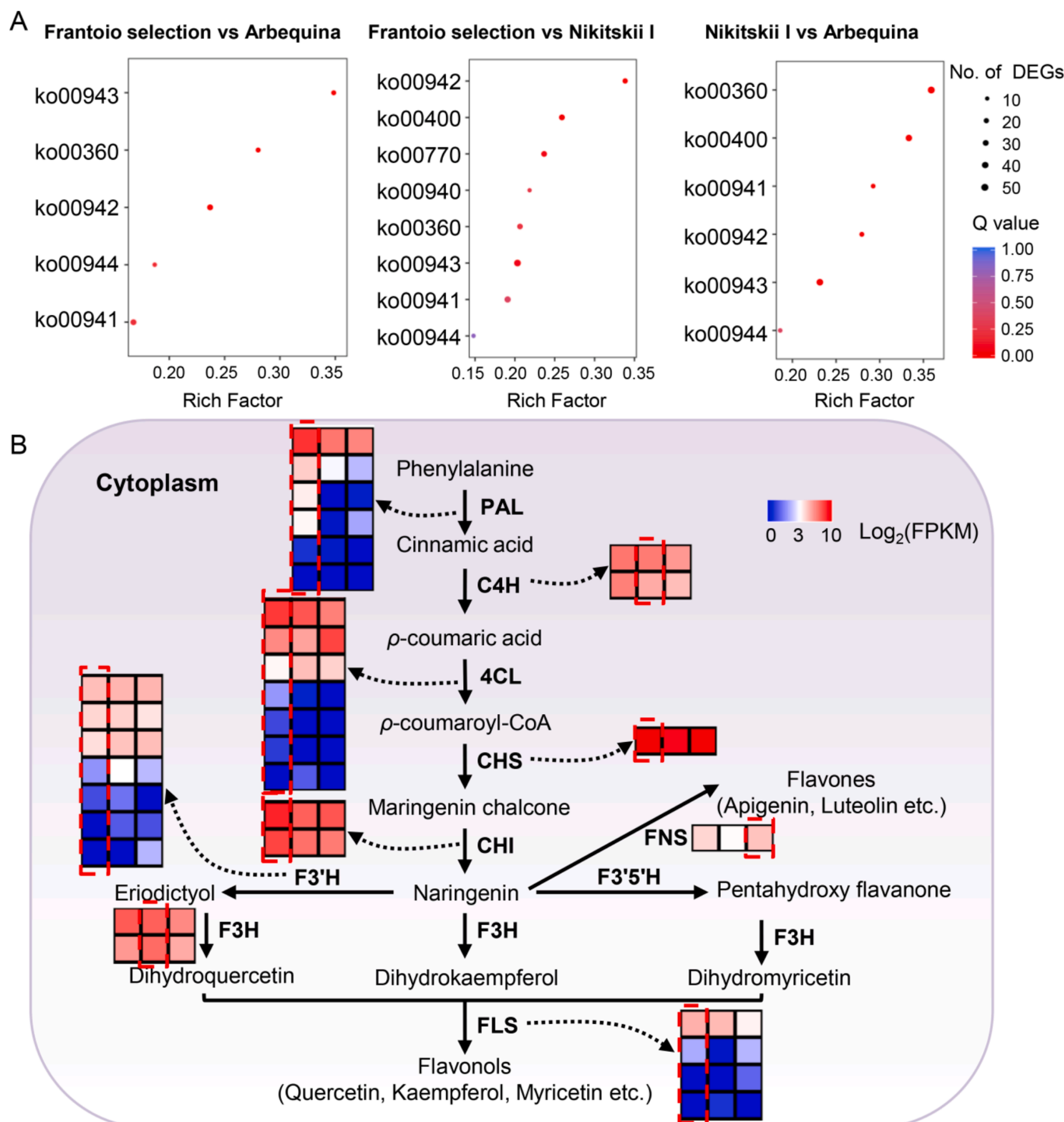

Fig. 5. Pathways and expression levels of differentially expressed genes (DEGs) related to flavonoid metabolism. (A) Kyoto encyclopedia of genes and genomes (KEGG) pathways related to flavonoid metabolism. The X-axis and Y-axis represent rich factors and different pathways. ko00943: Isoflavonoid biosynthesis; ko00360: Phenylalanine metabolism; ko00942: Anthocyanin biosynthesis; ko00944: Flavone and flavonol biosynthesis; ko00941: Flavonoid biosynthesis; ko00400: Phenylalanine, tyrosine and tryptophan biosynthesis; ko00770: Pantothenate and CoA biosynthesis; ko00940: Phenylpropanoid biosynthesis. (B) Molecular regulation and DEGs of flavonoid biosynthesis. The three cultivars were 'Arbequina', 'Frantoio selection' and 'Nikitskii I', and expression levels of DEGs were recorded as Log_2(FPKM). All transcripts of each type of protein were calculated in individual cultivars, and the cultivar with the highest transcripts was marked with a red dotted line. PAL: Phenylalanine ammonia-lyase; C4H: Cinnamate 4-hydroxylase; 4CL: 4-coumarate CoA ligase; CHS: Chalcone synthase; CHI: Chalcone isomerase; F3′H: Flavonoid 3′-hydroxylase; F3′5′H: Flavonoid 3′5′-hydroxylase; FNS: Flavone synthase; F3H: Flavanone 3-hydroxylase; FLS: Flavonol synthase (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

involved in coloration regulation and stress resistance (Chen et al., 2020; Ferrer et al., 2008). It is evident that the prolific flavonoid biosynthesis, pigment accumulation, and stress defense occurred in 'Arbequina'. Moreover, F3'H and FNS are responsible for the synthesis of different Flavonols and Flavones. Rutin is an important flavonol compound and also intermediate or precursor in Quercetin biosynthesis (Long et al., 2019; Iaria et al., 2016; Rao et al., 2019; Rossi et al., 2016). Although the total expression of F3'H was higher than that of FLS in each cultivar, the detected accumulation of Flavonols was less than Flavones (Fig. 5B, Table 3). These DEGs and regulatory network were firstly displayed in different olive cultivars, whether there are new or more complex metabolic pathways, and how they determine more active flavonoid synthesis in olive than in other oil plants need to be further studied.

4. Discussion

Olive (*Olea europaea* L.) has been cultivated around 6000 years in Mediterranean countries and has the universal adaptability to the weakly-alkaline soil and drought summer (Zohary and Hopf, 1994; Deng, 2018). When it was introduced in other countries in the 20th century, environmental factors such as acid soil and excessive rainfall severely hindered its expansion (Deng, 2018). Niu et al. (2021) have reported the identification of acid-tolerant cultivars suitable for growing in rainy environmental conditions for the first time. Based on the previous results, this study further characterized the morphological, agronomical traits and fatty acid or flavonoid metabolisms of 'Arbequina', 'Frantoio selection' and 'Nikitskii I' by multi-omics analysis.

Olive fruits have two main economic purposes: olive oil and table olive. Olive oil is the only woody oil extracted from the fresh fruits by physical-cold method, which preserves natural substances to the greatest extent. While table olive is one kind of fermented vegetables, treated with sodium hydroxide, salt or natural fermentation (Muzzalupo, 2012b). 'Arbequina' is a worldwidely grown cultivar for oil extraction purpose. In the present study, it showed a low fruit weight (1.79 g) with a spherical fruit shape and elliptic stone shape (Fig. 1, Table 1), which was consistent with that recorded in the OLEA database and World Catalogue of Olive Varieties (Barranco et al., 2000). However, the oil contents of 'Arbequina' (15.94%) were lower than that previously reported (>20%) (Barranco et al., 2000). Similar results also existed in 'Nikitskii I' (13.13%). Although it is hard to pinpoint the causes of the lower oil contents, excessive rainfall in summer coinciding with the oil accumulation phase is high speculated in this case. 'Nikitskii I' is a cultivar with biggest fruit size (4.18 g), biggest fruit flesh/pit ratio (6.25) and moderate fruit oil content (13.13%) (Fig. 1, Table 1), thus it could be used for both oil and table purpose. 'Frantoio selection' was a novel clone selected and the information was therefore rarely available in literature. Generally it was a cultivar for oil use with middle fruit size (2.78 g), ovoid fruit shape, moderate oil content (12.95%) and late maturity (Fig. 1, Table 1).

Olive oil is rich in unsaturated fatty acids and flavonoids, which is crucial to its quality. Different from the previous works, the fatty acid and flavonoid compounds were detected based on olive fruits rather than on olive oil (Bouymajane et al., 2020; Rizwan et al., 2019). As the result, the average Pearson correlation indexes of fatty acids and flavonoids were up to 0.985 and 0.971 among three cultivars, respectively. The contents of different fatty acid components of the three cultivars were in the same order as C18:1, C18:2, C16:0, C16:1, C18:0 and C18:3N3 from high to low, showing a high consistency (Table 2). The contents of C18:1, C18:2 and C16:0 were always the top three among all fatty acid compounds with C18:1 as the first as previously reported (Bouymajane et al., 2020; Rizwan et al., 2019). It implied that C18:1, C18:2 and C16:0, as the main fatty acid components, were relatively stable in olive regardless of the fruit/oil or cultivars or growing environment. Compared with the fatty acid compositions, the flavonoid compounds in different cultivars displayed a relatively lower consistency (Table 3), which was consistent with the results of previous studies (Bouymajane et al., 2020; Rizwan et al., 2019; Lukic et al., 2019). However, among the detected flavonoid compounds, Luteolin, Rutin, Cynaroside and Kaempferol were still the top four highest contents in these three cultivars.

On the whole, 'Frantoio selection' and 'Nikitskii I' displayed relatively bigger difference both in the fatty acid compounds and flavonoid compositions among the three cultivars, so as the differentially expressed genes (Tables 2, 3, Supplementary Fig. 2). In the process of fatty acid metabolism, except for SAD regulating the formation of C18:1, genes such as FAD2 and FAD3 that catalyze fatty acid desaturation showed lower expression levels. Among them, 'Frantoio selection' identified more up-regulated genes involved in FAs and UFAs biosynthesis (Fig. 4) and displayed the highest accumulation in UFAs/FAs (Table 2). Except for the expression of FAD3, 'Nikitskii I' had no obvious advantages in expression levels of most genes (Fig. 4), which just result to that more fatty acids existed in the form of monounsaturated fatty acid C18:1 instead of polyunsaturated fatty acids (Table 2). Although showing poor proportion of UFAs/FAs and C18:1/FAs, 'Arbequina' presented higher expression levels of genes related to flavonoid biosynthesis and had actually 2.19-time and 2.35-time that of 'Frantoio selection' and 'Nikitskii I' in the total flavonoid contents, respectively (Fig. 5 and Table 3). This may explained why 'Arbequina' fruit had higher flavonoid content than the other two cultivars.

5. Conclusion

The study identified the fruits traits of three olive cultivars, 'Arbequina', 'Frantoio selection' and 'Nikitskii I' first planted in the conditions of acid soil and rainy summer. In summary, 'Arbequina' and 'Frantoio selection' were suitable for oil extraction, while 'Nikitskii I' could be used both for table olives and olive oil purposes. The three cultivars showed the highest accumulation in total flavonoid contents, UFAs/FAs and C18:1/FAs, respectively, and 44/32 prior DEGs were detected involved in the fatty acid/flavonoid biosynthesis. At the morphological, agronomical, metabolomic and transcriptomic levels, the results clarified the economical purposes, special metabolites and related genes of three olive cultivars, aiming to provide references for cultivation and production of olive industry. By the traditional or molecular breeding, the metabolites and candidate genes in the individual cultivar would be further benefit for the genetic improvement of the quality traits of olive oil.

CRediT authorship contribution statement

Erli Niu: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Wenjun Hu:** Methodology, Formal analysis. **Jian Ding:** Methodology, Formal analysis. **Wei Wang:** Writing – review & editing. **Agustí Romero:** Writing – review & editing. **Guoxin Shen:** Writing – review & editing. **Shenlong Zhu:** Conceptualization, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.111017.

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