horticulturae

MDPI

Article

Widely Targeted Metabolomics Provides New Insights into the Flavonoid Metabolism in 'Kyoho' Grapes under a Two-Crop-a-Year Cultivation System

Guo Cheng [1,†], Sihong Zhou [1,†], Jinbiao Liu [1], Qiyan Feng [1], Rongfu Wei [1], Huan Yu [1], Bo Wang [2], Ying Zhang [1] and Xianjin Bai [1,*]

[1] Grape and Wine Research Institute, Guangxi Academy of Agricultural Sciences, Nanning 530007, China
[2] College of Agriculture, Guangxi University, Nanning 530004, China
* Correspondence: b5629@126.com
† These authors contributed equally to this work.

Abstract: The establishment and application of a two-crop-a-year cultivation system depends on the particularity of climatic conditions in subtropical regions. The different temperature, light, and water conditions throughout the growing season of summer and winter grapes are the fundamental reasons for differences in primary and secondary metabolites. We performed ultra-high-performance liquid chromatography–triple quadrupole mass spectrometry (UPLC-QQQ-MS)-based metabolomics on 'Kyoho' grapes under a two-crop-a-year cultivation system. In total, 1062 metabolites were identified and classified into 10 different categories, while flavonoids were the largest group, with 285 metabolites. Moreover, 876 metabolites were different among the four developmental stages, and 551 were different between the summer and winter grapes during the same growth period. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) clearly distinguished developmental and growth-season differences based on the detected metabolites. Of note, flavonoids were the most important compounds responsible for the differences in berry composition during the growth and developmental seasons. The content of most flavonoids was higher in the winter grapes, but some were also found at higher levels in summer grapes, such as kaempferol-4′-*O*-glucoside, leucocyanidin, and cyanidin-3-*O*-glucosylglucoside. Additionally, myricetin-3-*O*-arabinoside was consistently higher in winter grapes than in summer grapes during all four developmental stages. The extreme high temperature and higher relative humidity were important reasons for the lower flavonoid content in the summer grapes than in the winter grapes. Moreover, the stronger light intensity in the early development of the winter grapes had a positive effect on the accumulation of flavonoids, especially flavonols and flavan-3-ols. This study provides new insights into the metabolism of flavonoids in grapes under a two-crop-a-year cultivation system and explores the climatic causes of the differences in the metabolites in the two crops of grapes.

Keywords: widely targeted metabolomics; two-crop-a-year; flavonoids; grape

Citation: Cheng, G.; Zhou, S.; Liu, J.; Feng, Q.; Wei, R.; Yu, H.; Wang, B.; Zhang, Y.; Bai, X. Widely Targeted Metabolomics Provides New Insights into the Flavonoid Metabolism in 'Kyoho' Grapes under a Two-Crop-a-Year Cultivation System. *Horticulturae* **2023**, *9*, 154. https://doi.org/10.3390/horticulturae9020154

Academic Editor: Nikolaos A. Nikolaou

Received: 15 December 2022
Revised: 20 January 2023
Accepted: 22 January 2023
Published: 26 January 2023

1. Introduction

The southern tropical region of China is an area traditionally seen as unsuitable for cultivation, due to its insufficient low temperatures and hot rainy season [1,2]. It is generally difficult to promote sprouting in grapevines, there is a slow differentiation of flower buds and low fruit quality, and it is difficult to concentrate sales at maturity [3]. Since 2003, the research and application of two-crop-a-year cultivation technology system have solved the traditional problems above, and a special advantage viticulture area has been formed in Guangxi (a province in southern China) [3]. Grape two-crop-a-year cultivation technology involves promoting a second differentiation of flower buds, using the summer buds or winter buds, on green shoots to bloom in the same year or breaking the dormancy of the winter buds of the old ripe branches [3]. Grapes harvested in the first growing season

are called summer grapes, and grapes harvested in the second growing season are called winter grapes.

Flavonoids are important secondary metabolites in grapes and play a vital role in grapevine growth and development, resistance to ultraviolet light and insect diseases, fruit color, flavor quality, and nutritional value [4]. Anthocyanins, flavonols, and flavan-3-ols are the main flavonoids that accumulate in grape berries [5]. In addition to a variety of factors, flavonoid profile and content are also affected by climatic conditions and cultivation techniques, which interact with each other to regulate the flavonoid metabolism in grapes [4,6]. For the same cultivar, climate and weather conditions can play a very important role [7,8]. Light is the most important climatic factor affecting flavonoid metabolism, including its the intensity, quality, and photoperiod. In general, light increases the levels of flavonoids, particularly anthocyanins and flavonols [9]. Therefore, in practice, leaf removal and cluster bagging are usually used to control grapes' exposure to light [10,11]. Temperature is another important environmental factor affecting flavonoid synthesis. Generally, low temperature can induce the expression of flavonoid-synthesis-related genes and increase their accumulation [12]. However, high temperatures severely inhibit the synthesis of flavonoids [4]. Water status is the third important environmental factor affecting flavonoid synthesis. An appropriate water deficit increases the accumulation of anthocyanins and proanthocyanidins, improves fruit by reducing fruit volume, and directly upregulates the expression of flavonoid-synthesis-pathway-related genes [6]. Therefore, regulated deficit irrigation can be used as a strategy to increase flavonoid contents in grapes [13].

Unique climatic conditions have been the basis for the development of two-crop-a-year cultivation [14]. Sufficient light in the second half of the year and a large temperature difference between day and night in autumn and winter are more conducive to the accumulation of photosynthates, and the grapes have a higher anthocyanin content and better color and quality [15,16]. Previous studies by our team showed that winter grapes had higher TSS and TA than summer grapes [17]. We also used transcriptome sequencing technology to explore the differences in the metabolic pathways of carotenoids and phenylpropane/flavonoids between the two crops [14]. Furthermore, certain structural genes of the flavonoid metabolic pathway were more highly expressed in winter grapes at the mature stage [14]. A previous study showed that winter grapes were more abundant in phenolics and aroma substances than summer grapes [18]. However, there have been few reports on the detection of metabolites in grapes from two crops based on a high-throughput, highly sensitive, and wide-coverage analytical method.

The present research was designed to analyze the flavonoids in summer and winter grapes of 'Kyoho' (*Vitis labrusca* L. × *Vitis vinifera* L.) by UPLC-QQQ-MS. In particular, the effects of climatic factors on the flavonoids of grapes from two crops were analyzed. This paper provides insights into the metabolism of flavonoids in summer and winter grapes. This will lay a theoretical foundation for further research on the metabolic mechanisms behind grape flavor substances under the two-crop-a-year cultivation system.

2. Materials and Methods

2.1. Experimental Vineyard Conditions and Two-Crop-a-Year Viticulture Practices

The experiment was conducted in 2020 in the vineyards of the Grape and Wine Research Institute, Guangxi Academy of Agricultural Sciences, located in Nanning, Guangxi Province, China (22°51′29″ N, 108°14′33″ E). The self-rooted vines in the vineyard were eight-year-old 'Kyoho' grape vines, managed on a canopy frame with a single trunk [19]. The vine spacing was 2.0 m × 6.0 m in north–south-oriented rows and a rain shelter cultivation mode was used. Daily management of the vineyard, including pest and water control methods, was carried out as per the previous standards for two crops per year [20].

The main cultural practices of two-crop-a-year cultivation are as follows (Figure 1A): There is medium cane pruning (4–7 buds) in January, followed by breaking dormancy using 50% hydrogen cyanamide diluted 20 times in late January to mid-February, when the temperature is stably above 10 °C. Flower thinning is carried out in late March to early

April, and harvesting of summer grapes (Figure 1B) occurs in mid-June to early July. Then, fertilization is carried out after the summer grape harvest, to restore the vines' growth. Long-cane pruning (8–10 buds) occurs in late July to mid-August and, at the same time, all of the leaves are removed and dormancy is broken using 50% hydrogen cyanamide diluted 30 times. Then, 5–8 days after germination, the second growth cycle of the year begins, and the harvesting of winter grapes (Figure 1C) takes place in late December to mid-January.

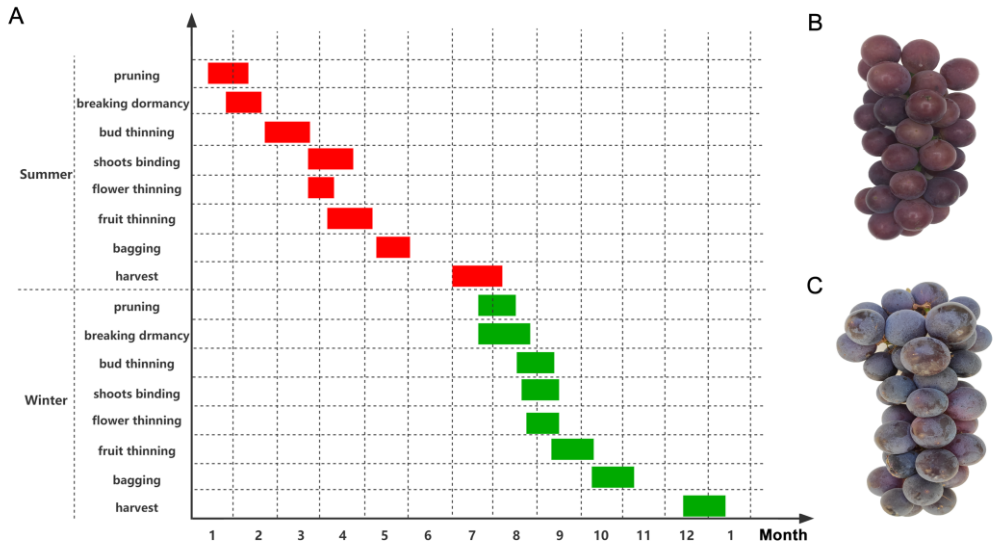

Figure 1. Cultural practices of two-crop-a-year cultivation (**A**). Summer grape (**B**) and winter grape (**C**) at harvest time. Specific period of cultivation practices for the production of summer and winter grapes are provided by red and green rectangles, respectively.

2.2. Meteorological Survey

The meteorological data during berry development in 2020, including the sunlight duration (h), temperature (°C), relative humidity (%), and solar radiation intensity (W/m^2), were gathered using a Hobo temp/RH/SR smart sensor (Onset Computer Corporation, Bourne, MA, USA). Measurements were performed at 5 min intervals.

2.3. Sampling Method and Physical and Chemical Index Monitoring

Six hundred berries were sampled at four Eichhorn–Lorenz stages [21]: berries still hard and green (E-L 33), the onset of veraison (E-L 35), berries not quite ripe (E-L 37), and the harvest stage (E-L 38). Each biological replicate contained 9 vines, and 200 berries were randomly separated from each biological replicate. Immediately, 100 berries were randomly selected to determine the color parameters, berry fresh weight, soluble solids concentration (SSC), and titratable acidity (TA). The color parameters were determined with a colorimeter (Konika Minolta CR-10, Japan). These included brightness value (L^*), red–green tone (a^*), and yellow–blue tone (b^*). Then, the berry was crushed to extract the juice. The SSC of the juice was measured with a digital, pocket-sized, handheld refractometer (Digital Hand-held Pocket Refractometer PAL-1, Atago, Tokyo, Japan). The TA was determined by acid–alkali titration with NaOH, the end point was pH 8.2, and the TA content was expressed as the tartaric acid equivalent. The remaining berries were immediately frozen in liquid nitrogen for metabolomics analysis.

2.4. Widely Targeted Metabolomics Methods

2.4.1. Sample Preparation and Metabolite Extraction

First, 24 frozen grape samples were freeze-dried using a vacuum freeze-dryer (Scientz-100F, Ningbo, China). The dried sample was ground using a mixer mill (MM 400, Retsch, Shanghai, China) for 1.5 min at 30 Hz. The extraction process of the samples was carried out as detailed by Yang et al. [13]. Specifically, 100 mg of powder for each sample was weighed

and dissolved in 1.0 mL of extracting solution (70% aqueous methanol). The extraction time was 24 h at 4 °C. Then, the mixtures were centrifuged at 10,319× *g* for 10 min, and the supernatants were isolated and filtered (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China) before UPLC-ESI-MS/MS analysis.

2.4.2. UPLC Conditions

The sample extracts were analyzed using an LC-ESI-MS/MS system (HPLC: Shimpack UFLC Shimadzu CBM30A system, www.shimadzu.com.cn/, accessed on 2 September 2021; MS: Applied Biosystems 6500 Q TRAP, www.appliedbiosystems.com.cn/, accessed on 2 September 2021). The 24 grape samples were analyzed under the following UPLC conditions based on a previous report [22]: column, Agilent SB-C18 (1.8 μm, 2.1 mm × 100 mm). The components of mobile phase A were 0.1% formic acid and pure water. The components of mobile phase B were 0.1% formic acid and acetonitrile. The A:B (*v*:*v*) gradient program was as follows: 95:5 (*v*:*v*) at 0 min, 5:95 (*v*:*v*) at 9.0 min, 5:95 (*v*:*v*) at 10.0 min, 95:5 (*v*:*v*) at 11.1 min, and 95:5 (*v*:*v*) at 13.0 min. The flow rate was maintained at 0.35 mL/min. The column oven was set to 40 °C, and the injection volume was 4 μL. The effluent was alternatively connected to an ESI triple quadrupole linear ion trap (QTRAP)-MS.

2.4.3. ESI-Q TRAP-MS/MS

We used a triple quadrupole linear ion trap mass spectrometer (Q TRAP), AB4500 Q TRAP UPLC/MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive ion mode, and controlled by Analyst 1.6.3 software (AB Sciex, Framingham, MA, USA). The ESI source operation parameters were selected according to previous reports [13]. The specific MRM transitions in each period were monitored based on the metabolite elution during this period.

2.5. Statistical Analysis

2.5.1. PCA and HCA

PCA was performed to preliminarily understand the overall metabolic difference between the 24 grape samples, using the statistics function prcomp within R (www.r-project.org, accessed on 2 September 2021). The data were unit variance scaled before unsupervised PCA. The analysis results of the HCA are shown in the form of heatmaps with dendrograms. Both PCA and HCA were carried out using the R package pheatmap.

2.5.2. Differential Metabolite Analysis

The significantly differential metabolites obtained by pairwise comparison were screened using the following conditions: VIP ≥ 1 and absolute log2FC (fold change) ≥ 1. The VIP values were extracted from the OPLS-DA results, which also contained score plots and permutation plots, generated using the R package MetaboAnalystR. The data were log transformed (log2) and mean centered before OPLS-DA. In order to avoid overfitting, a permutation test (200 permutations) was performed.

2.5.3. Kyoto Encyclopedia of Genes and Genomes (KEGG) Annotation and Enrichment Analysis

The identified metabolites were annotated using the KEGG Compound database (http://www.kegg.jp/kegg/compound/, accessed on 2 September 2021); then, all of the annotated metabolites were mapped to the KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html, accessed on 2 September 2021). The pathways with significant regulatory metabolites were input into MSEA (metabolite sets enrichment analysis), and their significance was determined using the *p*-values of the hypergeographic test.

3. Results and Analysis

3.1. Meteorological Characteristics

The cultivation mode of two crops per year makes full use of the temperature and light resources in the second half of the year in the South Asian tropical area to produce winter grapes. Table 1 displays the large differences in the phenological periods and climatic conditions between the summer and winter grape growing stages. The growing season for summer grapes is from late February to late June, and the winter grape season is from early September to late December. In general, the time from E-L 4 (first leaf tissue being visible) to E-L 35 (veraison) of the summer fruit was longer than that of the winter fruit, especially from E-L4 to E-L 19 (the beginning of flowering). However, the winter grapes took 18 days longer than the summer fruits from E-L 35 to E-L 38 (harvest–ripeness). For the summer grapes, there were 27 days of high temperatures over 35 °C from E-L 35 to E-L 38. The active accumulated temperature and the effective accumulated temperature of the summer grapes were higher than those of winter grapes with the exception of E-L 19 to E-L 26 (cap fall complete). Moreover, the relative humidity during the summer growing season showed a higher value than during the winter growing season. During the second growing season, the solar radiation intensity was higher than for the first crop from E-L 4 to E-L 26. However, the result was the opposite for the winter crop from E-L 26 to E-L 38.

Table 1. Phenology and climatic factors during the two crop-growing seasons in Nanning in 2020.

Meteorological Data	E-L 4–19		E-L 19–26		E-L 26–35		E-L 35–38	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Phenology	27 February–30 March	5 September–22 September	31 March–7 April	23 September–28 September	8 April–26 May	29 September–6 November	27 May–29 June	7 November–28 December
Number of days	33	18	8	6	49	39	34	52
Number of days ≥35 °C	0	12	0	1	0	1	27	0
Active T [a] (°C)	686.08	518.12	131.73	155.48	1269.39	913.93	1015.791	901.49
Effective T [b] (°C)	356.08	338.12	51.73	95.48	779.39	523.93	675.79	381.49
Relative humidity [c] (%)	85.71	80.95	91.63	84.44	77.87	77.59	80.50	75.69
Solar radiation Intensity [d] (W/m^2)	52.68	110.37	37.05	80.71	108.44	89.64	132.20	73.39

[a] Active accumulated temperature for each phenology of the summer and winter crops, calculated as T = ∑ti (ti ≥ 10 °C); ti is the average daily temperature. [b] Effective accumulated temperature for each phenology of the summer and winter crops, calculated as T = ∑(ti − 10) (ti ≥ 10 °C); ti is the average daily temperature. [c] Relative humidity is the daily average value for each phenology of the summer and winter crops. [d] Solar radiation intensity is the daily average value for each phenology of the summer and winter crops.

3.2. Physical and Chemical Indexes of Summer and Winter Grapes

In order to compare the difference in the physical and chemical indexes between the summer and winter grapes at different developmental stages, we measured the berry fresh weight, SSC, TA, and color characteristics. Compared with the winter grapes, the summer grapes showed a significantly higher berry fresh weight in each of the four stages (Figure 2A). In addition, compared with the summer grapes, the winter grapes showed a significantly greater SSC at E-L 38, but no significant difference in the other stages (Figure 2B). The TA content of the winter grapes was significantly higher than that of summer grapes during the entire developmental stage (Figure 2C). The color attributes of the grapes from the two crops showed that the value of L^* and b^* gradually decreased (Figure 2D,F), and the a^* value gradually increased (Figure 2C). Furthermore, the L^* value of summer grapes during the entire ripening stage was significantly higher than that of the winter grapes, which indicated that the winter grapes were darker in color (Figure 2D). On the other hand, the a^* and b^* values of the winter grapes at the later stage of maturation were

significantly higher than those of the summer grapes (Figure 2E,F). This suggested that the red and yellow tonalities of the winter grapes was weaker than those of the summer grapes.

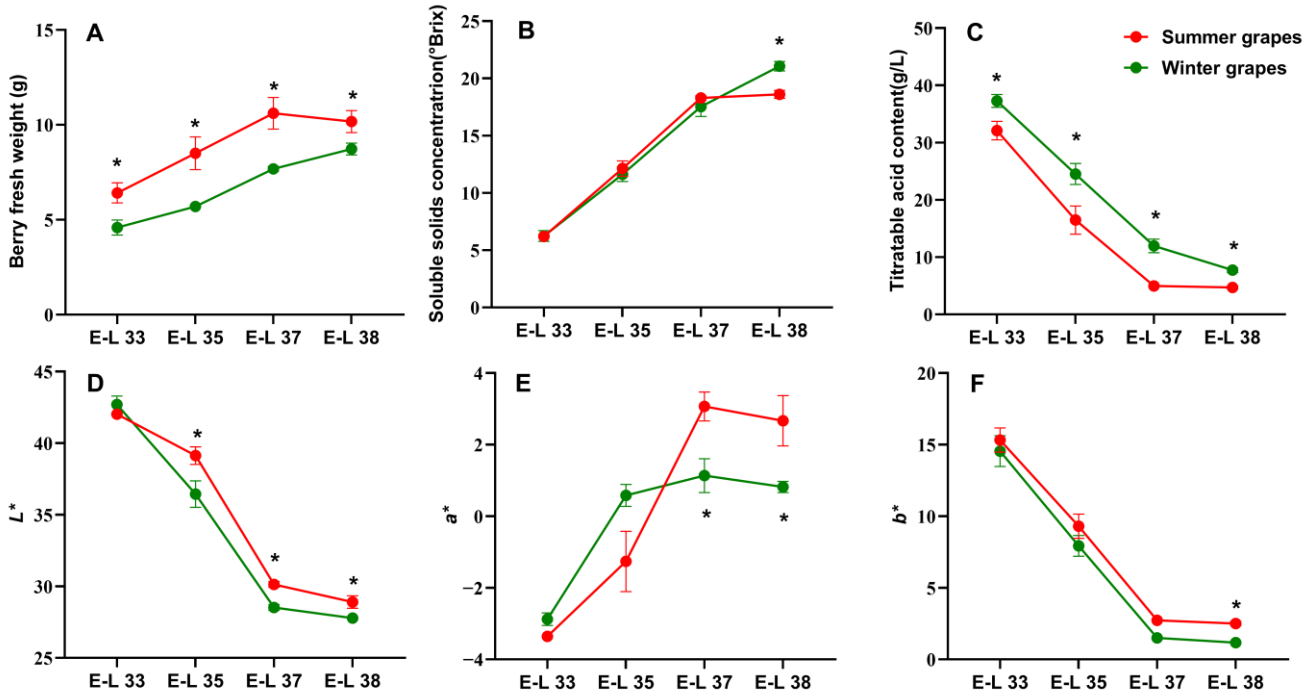

Figure 2. Evolution of the berry fresh weight (**A**), SSC (**B**), TA (**C**), *L** (**D**), *a** (**E**), and *b** (**F**) for the two crops of 'Kyoho' grapes in 2020. The four points refer to stages E-L 33, 35, 37, and 38, respectively. The asterisk indicates significant differences between the summer and winter 'Kyoho' grapes at the same stage.

3.3. Overview of the Metabolites of the Grapes from the Two Crops

We examined the metabolite characteristics in the four developmental stages, especially the difference between the summer and winter grapes. The widely targeted metabolomic analysis revealed 1062 metabolites (Table S1) in this study, including flavonoids (285), phenolic acids (129), lipids (122), amino acids and derivatives (108), organic acids (66), alkaloids (58), nucleotides and derivatives (54), terpenes (52), tannins (28), and lignans and coumarins (26) (Figure 3A). In order to clarify the different metabolites during the same growing season and between the two crops at the same developmental stage, the number of upregulated and downregulated metabolites was counted, as shown in Figure 3B. The results of the pairwise comparison among the green-fruit stage (E-L33) and the mature stages (E-L35, E-L37, and E-L38) were similar for the summer and winter grapes. Specifically, the number of downregulated metabolites was higher than that of the upregulated metabolites. For the pairwise comparison between the mature stage samples, the results of the summer fruits and winter fruits were different. First, the total number of differential metabolites was higher in the summer fruits than in the winter fruits. Second, the number of upregulated metabolites was higher than those downregulated in the pairwise comparison of the winter fruits during the ripening stage. Of note, most metabolites were upregulated in the comparison between the winter fruits and the summer fruits at the same developmental stage. In addition, the number of differential metabolites in the three mature stages was higher than those in the green-fruit stage.

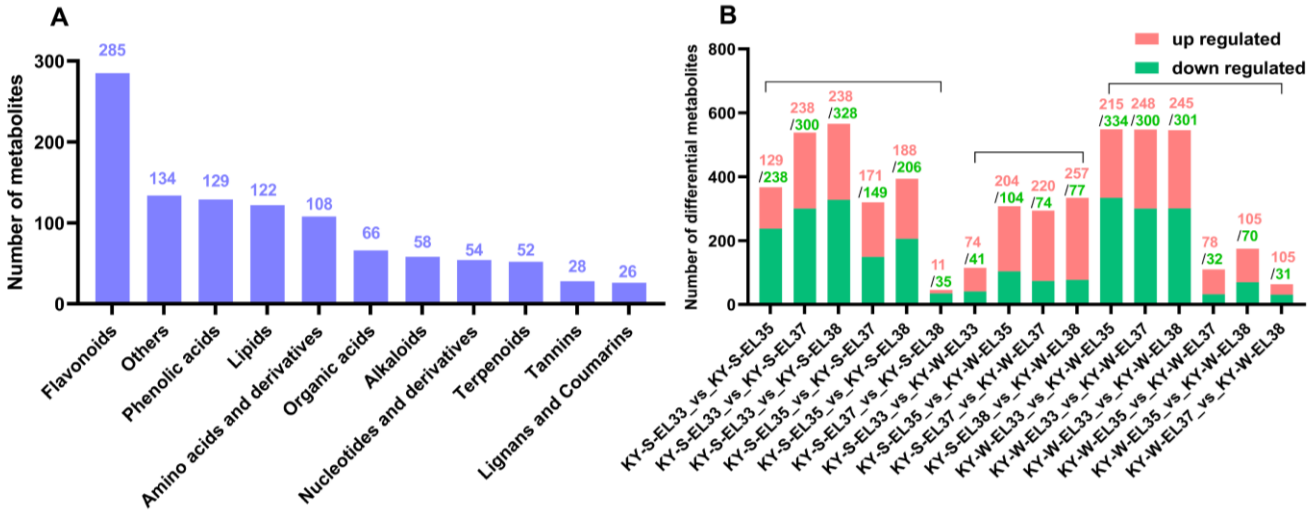

Figure 3. Categorical metabolite statistics (**A**) and pairwise comparison of differential metabolites (**B**).

The comparison results of all metabolites among the different samples are comprehensively shown in Figure 4A, and these metabolites were divided into two groups through horizontal clustering. Metabolites in the first group preferentially accumulated in the maturing stages of summer and winter grapes, and the metabolites in the second group accumulated more in the green-fruit stage. The PCA divided the 24 samples into three groups, and the results were consistent with the cluster dendrogram (Figure 4B). The results revealed that the composition and content of metabolites in the green-fruit stage were significantly different from those in the mature stages. Meanwhile, there were also significant differences between the summer and winter fruits in E-L35, E-L37, and E-L38.

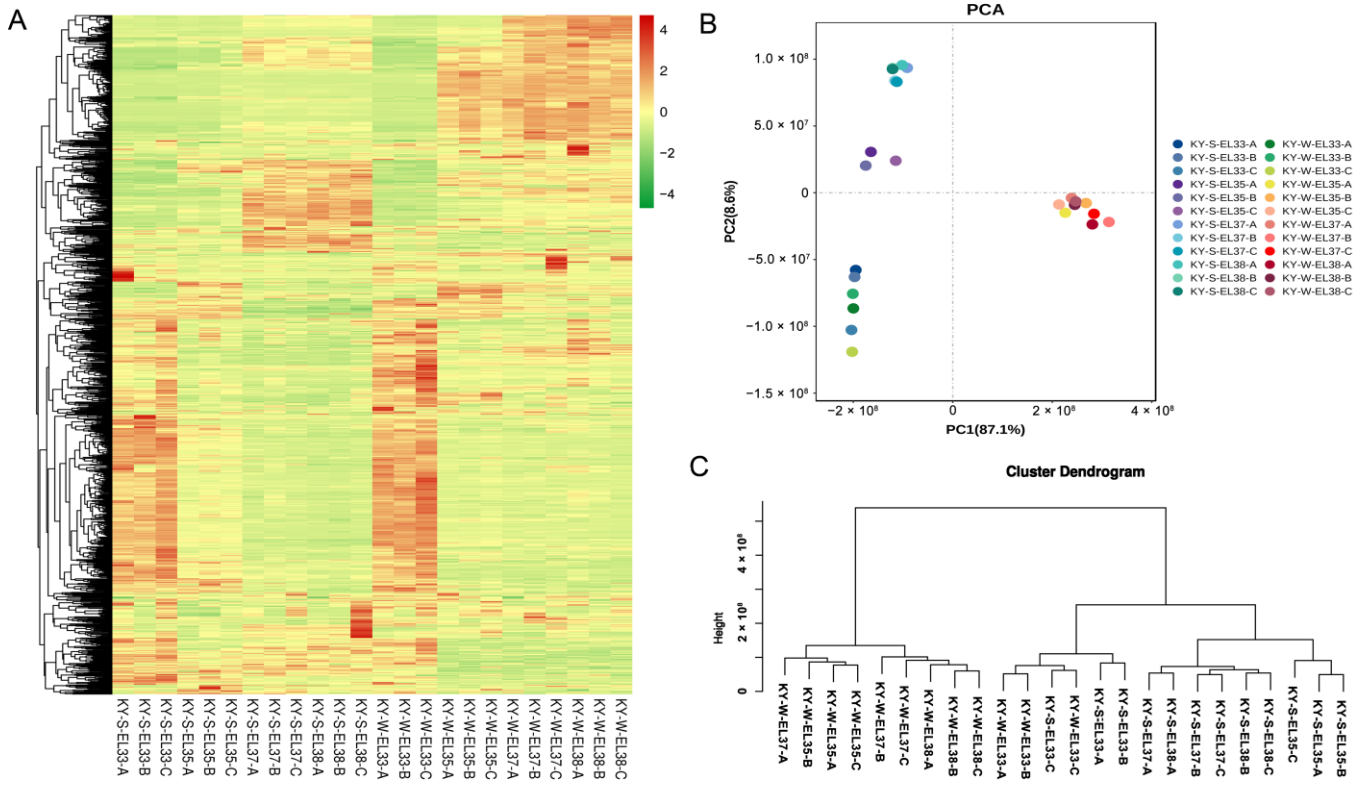

Figure 4. Overview of the 1062 metabolites in 24 'Kyoho' grape samples containing biological duplicates (**A**); principal component analysis (PCA) (**B**); cluster dendrogram of metabolome data from all grapes (**C**).

3.4. Differential Accumulation of Metabolites

In order to more clearly analyze the metabolite accumulation characteristics in the developmental periods, a K-means clustering analysis was conducted for the summer and winter grapes. The 876 differential metabolites in eight grape samples were clustered into five subclasses, based on the metabolic profiling differences (Figure 5). A total of 484 metabolites in subclasses 1 and 2 showed a higher content in summer and winter grapes from E-L33 (Figure 5A,B). The content of 392 metabolites in subclasses 3, 4, and 5 was higher in grapes from E-L35, E-L37, and E-L38 (Figure 5C–E). Overall, flavonoids presented the highest proportion in each subclass; in particular, the proportion was as high as 64% (Figure 5F) in subclass 4.

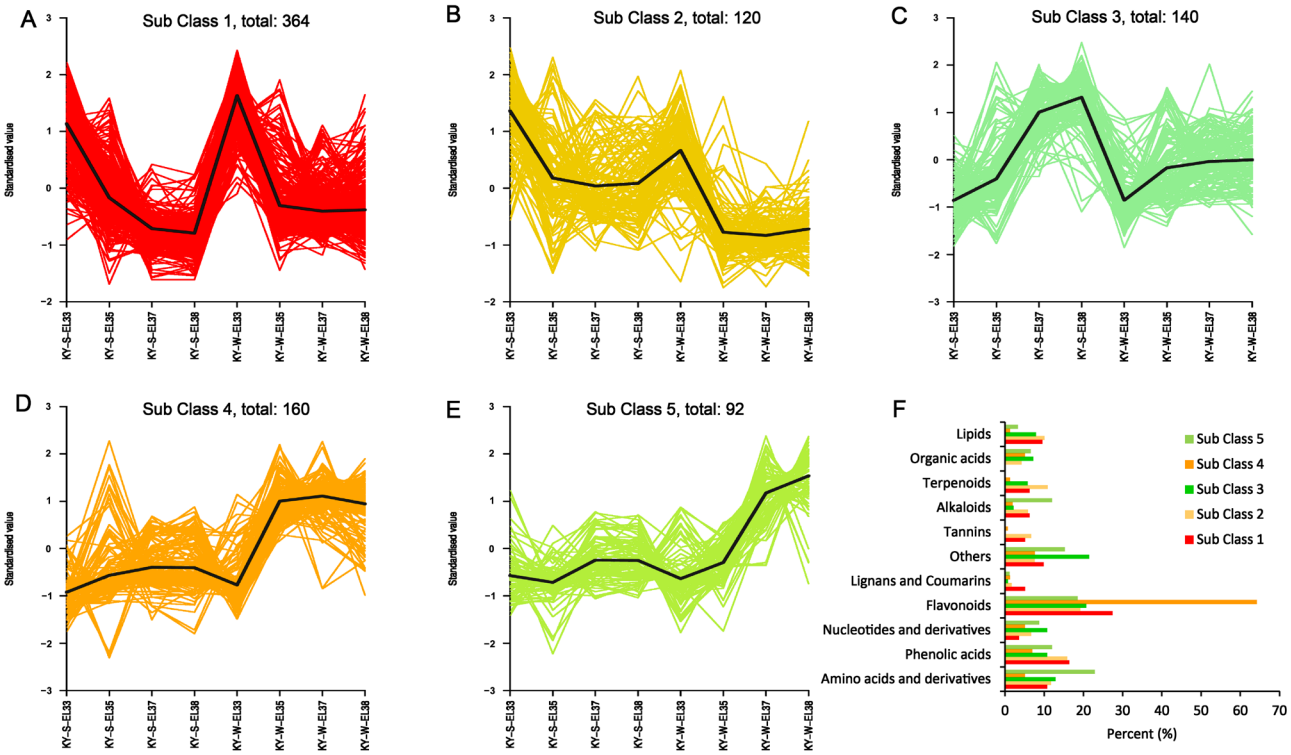

Figure 5. K-means clustering analysis of the patterns of 876 differential metabolites in 'Kyoho' grapes from the two crops. Sub Class 1-5 (**A**–**E**); The metabolites percents for each subclass (**F**).

The Venn diagrams illustrate the relationships among the differential metabolites at the different developmental stages (Figure 6A): 38, 111, 22, and 63 differential metabolites were specifically detected in E-L 33, 35, 37, and 38, respectively. This indicates that the most unique metabolites were present in the summer or winter grapes at veraison (E-L 35). Only 24 differential metabolites were shared by all four stages (Table S2), and the proportion of each class is shown in Figure 6B. Specifically, the highest proportion was found for flavonoids, followed by phenolic acids. Myricetin-3-*O*-arabinoside was one of the flavonols that was consistently higher in winter grapes than in the summer counterparts. In addition, the results of the pairwise comparison between the green-fruit stage and the mature stage in the same growing season are shown in Figure 6C,D. Of note, 271 and 410 differential metabolites were shared by the three comparison groups for the summer and winter grapes, respectively. It was inferred that most of the differential metabolites were present in the whole process of fruit development for the winter grapes.

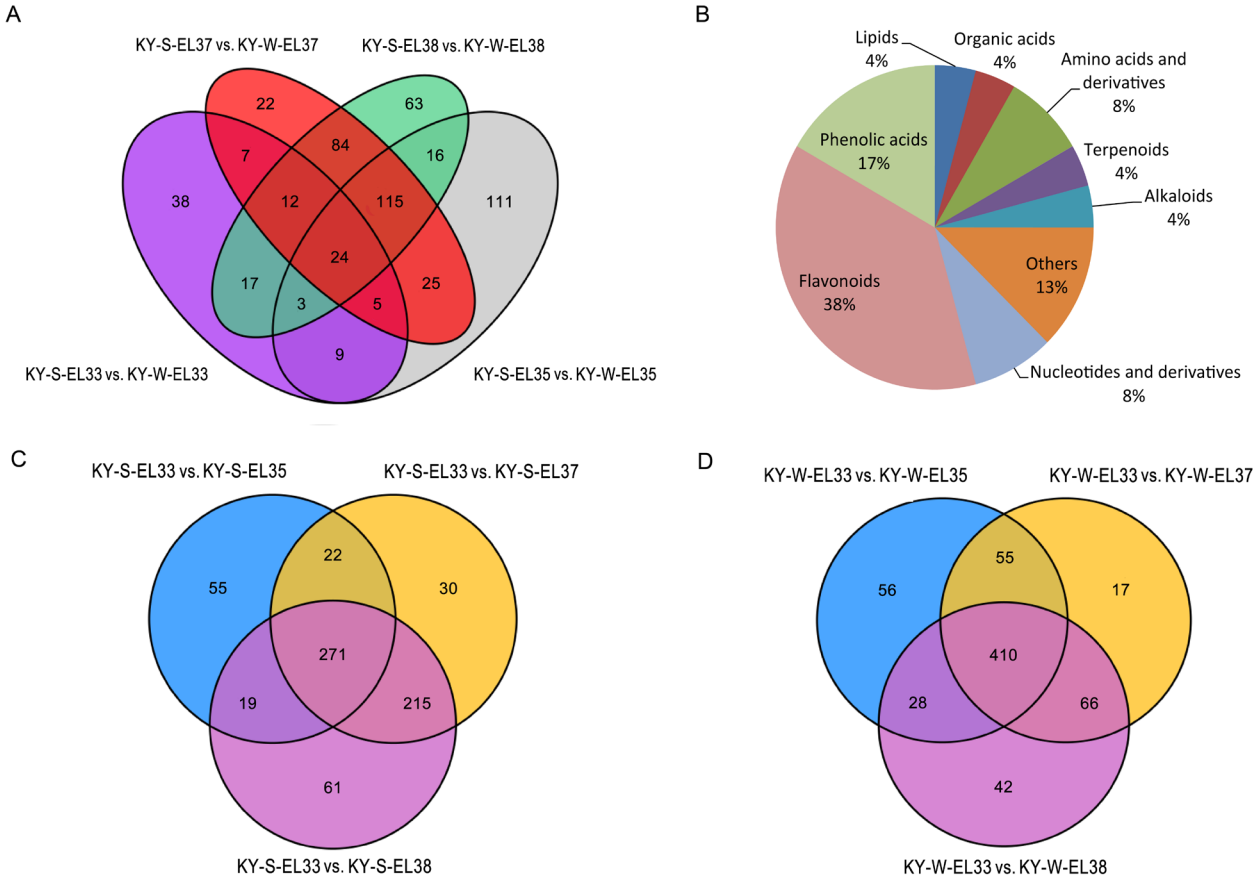

Figure 6. Venn diagrams of the differential metabolites for the four comparison groups (**A**); the proportion of 24 shared metabolites (**B**); Venn diagrams of the differential metabolites for summer 'Kyoho' grapes (**C**); Venn diagrams of the differential metabolites for winter 'Kyoho' grapes (**D**).

In order to find the metabolites with large differences between the two comparison groups, we ranked the top 10 substances using Log_2FC, including upregulation and downregulation. There were clear differences between the summer and winter grapes at the various developmental stages. Specifically, the upregulated and downregulated substances with the largest Log_2FC for KY-S-EL33_vs_KY-W-EL33 were ditartaroyl-hydroxycoumarin and erythorbic acid, respectively (Figure 7A). For KY-S-EL35_vs_KY-W-EL35, the upregulated and downregulated substances with the largest Log_2FC were patuletin-3-*O*-rutinoside and diosmetin-7-*O*-Beta-D-glucopyranoside (Figure 7B), respectively. It is worth noting that KY-S-EL37_vs_KY-W-EL37 and KY-S-EL38_vs_KY-W-EL38 showed similar comparisons. For example, the upregulated and downregulated substances with the largest Log_2FC were 4-*O*-beta-D-glucosyl-4-coumaric acid and diosmetin-7-Beta-D-glucopyranoside (Figure 7C,D).

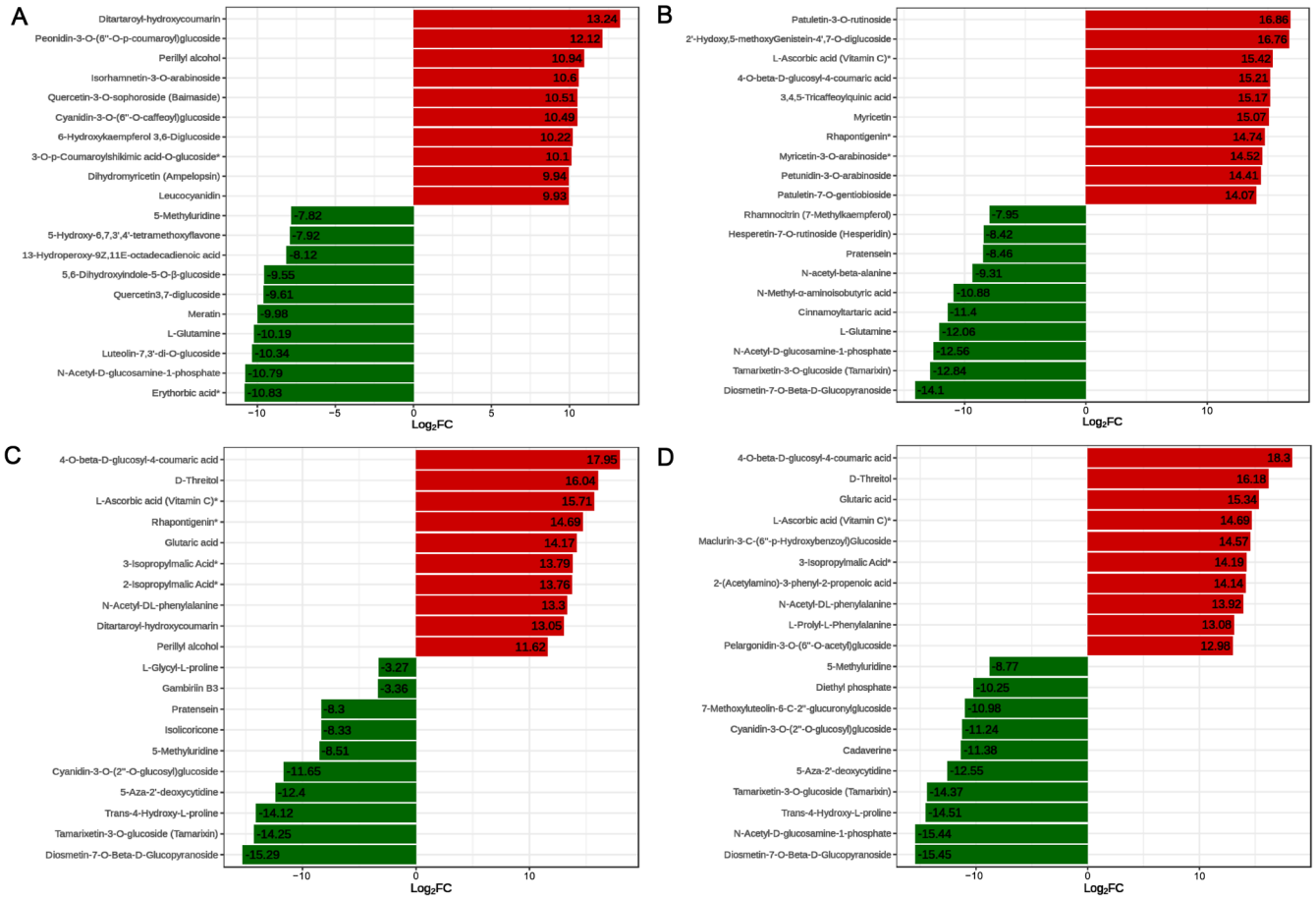

Figure 7. The top 10 differential metabolites between the summer and winter 'Kyoho' grapes from the same developmental stage. KY-S-EL33_vs_KY-W-EL33 (**A**); KY-S-EL35_vs_KY-W-EL35 (**B**); KY-S-EL37_vs_KY-W-EL37 (**C**); KY-S-EL38_vs_KY-W-EL38 (**D**). Upregulation and downregulation are provided by red and green bars, respectively.

3.5. Differential Metabolism of Flavonoids in Grapes from Two Crops

From the above analysis, it can be seen that flavonoid compounds mainly caused the differences between the comparison groups. Therefore, we focused on the analysis of the 136 differentially metabolized flavonoid compounds in the 24 samples (Figure 8). Specifically, these included 95 flavonols, 12 flavan-3-ols, and 29 ancyanidins. For flavonols, the content of most compounds was higher in the late ripening stage of the summer and winter grapes than in E-L33 and E-L35, mainly including certain kaempferols, quercetins, and isorhamnetins, and all myriceones, limocitrins, and patuletins. In addition, the content of these compounds in the winter grapes was higher than in the summer grapes. Of note, the content of some compounds in the late ripening stage of the summer grapes was higher than that of the winter fruits, such as kaempferol-4′-*O*-glucoside, kaempferol-3-*O*-rutinoside-7-*O*-glucoside, 6-methoxykaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-arabinoside, isorhamnetin-3-*O*-glucoside, and isorhamnetin-7-*O*-glucoside. For the flavan-3-ols, the content of most compounds was higher in the E-L33 stage than veraison and the late ripening stage of the summer and winter grapes. Furthermore, the content of these flavan-3-ols in the winter fruits was higher than in the summer fruits, except leucocyanidin. However, the content of leucocyanidin was higher in the late ripening stage of the summer grapes. The content of anthocyanins gradually accumulated in the summer and winter grapes over the developmental periods. Additionally, the content of most anthocyanins in the winter grapes at the ripening stage was higher than that in other samples, except cyanidin-3-*O*-glucosylglucoside and malvidin-3-*O*-acetylglucoside-5-*O*-glucoside.

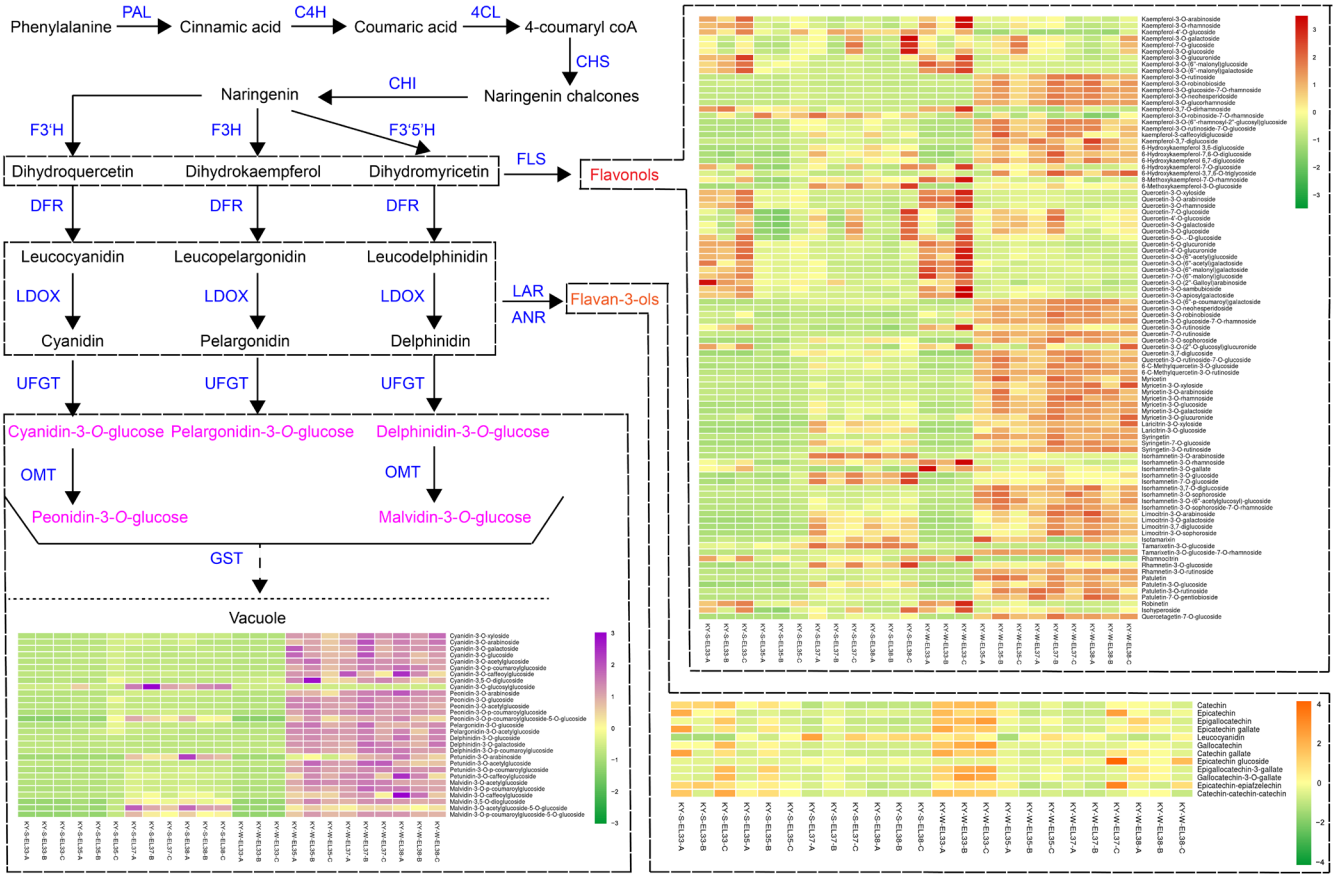

Figure 8. Profile of the differential metabolites involved in the flavonoid biosynthetic pathways in the summer and winter 'Kyoho' grape berries.

4. Discussion

In recent years, the widely targeted metabolomics approach has been used to detect metabolite characteristics in a variety of fruit plants, such as citrus [23], loquat [24], longan [25], kiwifruit [26], apple [27], mango [28], and grape [26]. The number of metabolites obtained using the widely targeted metabolomics approach is generally between 200 and 1000, which is very important for studying the characteristics of the metabolite components of different varieties. In 2022, a total of 774 metabolites were detected using the widely targeted metabolomics approach in two wine grape varieties, and there were 157 types of flavonoids in 400 secondary metabolites [22]. In the present study, 1062 metabolites were detected in summer and winter 'Kyoho' grapes, including 285 flavonoids, 129 phenolic acids, 122 lipids, 108 amino acids and derivatives, 66 organic acids, 58 alkaloids, 54 nucleotides and derivatives, 52 terpenes, 28 tannins, and 26 lignans and coumarins. According to the above analysis, there were 285 flavonoids in 578 secondary metabolites from 'Kyoho' grapes. Therefore, flavonoids accounted for a large proportion of all metabolites in both table and wine grapes. Moreover, a total of 25 types of anthocyanins were detected in the 'Kyoho' grape using the traditional HPLC-MS technique [29]. The 37 anthocyanins identified in all samples were verified using UPLC-QQQ-MS in our study.

Of the 1062 detected metabolites, 876 were different among the four developmental stages and 551 were different between the summer and winter fruits in the same period. Interestingly, both the developmental and the two-crop grape comparisons showed that the dominant class of differential metabolites was flavonoids. Flavonoids mainly consist of flavonols, flavan-3-ols, and anthocyanins in grapes, and they showed accumulation characteristics of variety, specificity, and developmental specificity [4,5]. Among them, flavonols were synthesized and accumulated in the skins during the early and mature

stages, flavan-3-ols were synthesized and accumulated in the skin and seeds during the early stage of fruit development, and anthocyanins were synthesized and accumulated in the pericarp from veraison to full maturity [30]. In the present study, the changes in the three types of flavonoids during development also conformed to the above research and were consistent with the differential gene expression of the two-crop grapes obtained in the phenylpropane-flavonoid synthesis pathway by our research group [14].

Compared with wine grapes, research on table grape metabolites has focused more on sugars, acids, and aroma substances [31,32]. As a classic red table grape, there is much research on the anthocyanin content of the 'Kyoho' grape [12,29,33]. However, few studies have comprehensively analyzed the composition characteristics of the phenolic substances, especially flavonoids. Sugar plays an important role in the synthesis of flavonoids especially anthocyanins in grapes, which can promote anthocyanin accumulation [34]. In our study, the contents of most anthocyanins and the concentration of soluble solids in winter grapes were higher than in summer grapes. Therefore, it is speculated that the insufficiency of sugars in summer grapes may be an important factor affecting anthocyanin synthesis. Previous studies have found that high temperature (>35°C) can promote anthocyanin degradation by inhibiting the expression of genes related to flavonoid metabolism, affecting enzyme activity and reducing ABA and flavonoid precursor (phenylalanine) contents [4]. Similar to anthocyanins, high temperature inhibited the expression of the key genes ANR and LAR for flavan-3-ols [35]. For the two-crop-a-year cultivation system, there were twice as many days of extreme high temperatures for the summer growing season than for the winter growing season. This is undoubtedly an important reason why the flavonoid content in the summer grapes was lower than in the winter grapes, and anthocyanins were more prominent than flavonols and flavan-3-ols. Light also plays an important role in flavonoid synthesis and accumulation and often has a synergistic effect with temperature [36,37]. UVB and UVC radiation can promote the accumulation of flavonols and flavan-3-ols in grapes [38,39]. CRY2 and HY5/HYHs, as light signaling components; MYBA1, as a transcription factor; and LAR, ANR, and FLS, as key structural genes, were responsible for the light-responsive biosynthesis of the corresponding flavonoids [11]. The light intensity in the early stage of the winter grapes was higher than that of the summer grapes, which could be an important reason behind more flavonols and flavan-3-ols being accumulated in the winter grapes. Moreover, water not only affects the physical properties of grapes but also has an important effect on the internal metabolites. In our study, the relative humidity of the winter grapes in the growing season was lower than that of summer grapes, and the drier environment could also cause a higher flavonoid synthesis. Lower humidity can promote the accumulation of flavonoids by reducing the berry fresh weight or by directly upregulating flavonoid-synthesis-pathway-related gene expression [6,8].

5. Conclusions

In the present study, the widely targeted metabolomics approach was implemented to systematically evaluate the difference in metabolites between 'Kyoho' grapes from two crops and during different stages. A total of 1062 metabolites were detected, 285 of which were flavonoids. Through clustering and differential metabolite distribution analysis, flavonoids were determined to be the most important group of differentiated metabolites in grapes between the two crops and between the four developmental stages. Overall, the differences between the developmental stages were greater than those between the summer and winter grapes at the same developmental stage. In total, 876 differential metabolites were identified in the four developmental stages, and 551 differential metabolites were identified in the summer and winter grapes during the same period. Furthermore, the metabolite composition and content in the green-fruit stage were significantly different from those in the mature stages. However, the number of differential metabolites in the mature stages was higher than in the green-fruit stage. The different climatic conditions in the summer and winter grape growing seasons were responsible for the difference in flavonoid metabolites between the two crops of grapes. Specifically, the larger number

of extreme high temperature days and the higher relative humidity in the summer grape growing season resulted in the lower flavonoid content of the summer grapes compared with the winter grapes. Furthermore, the accumulation of a larger number of flavonols and flavan-3-ols in winter grapes might have be related to the stronger light intensity in the early fruit development. Myricetin-3-*O*-arabinoside was consistently higher in winter grapes than the summer grapes during all developmental stages. Of note, most flavonoids had a higher content in winter grapes, but some were also higher in summer grapes, such as kaempferol-4′-*O*-glucoside, leucocyanidin, and cyanidin-3-*O*-glucosylglucoside. In summary, the present research detected the primary and secondary metabolites of 'Kyoho' grapes using a UPLC-QQQ-MS-based metabolomics approach. Additionally, the results revealed the climatic factors responsible for the difference in metabolites between the two seasons of summer and winter grapes. Future research may concentrate on the mechanisms by which climatic factors regulate quality-related metabolites under the two-crop-a-year cultivation system. In addition, through the study of specific cultivation techniques, it is hoped that we can overcome some defects in summer and winter grapes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9020154/s1, Table S1: All metabolites identified in the summer and winter 'Kyoho' grapes. Table S2: The 24 metabolites shared by the pairwise comparisons from all four stages.

Author Contributions: Conceptualization, G.C., S.Z., B.W. and X.B.; methodology, G.C., S.Z. and B.W.; software, G.C. and S.Z.; validation, G.C. and S.Z.; formal analysis, G.C., S.Z. and Q.F.; investigation, G.C., S.Z., J.L., Q.F., R.W. and H.Y.; resources, J.L., R.W., Y.Z. and X.B.; data curation, G.C., S.Z. and Q.F.; writing—original draft preparation, G.C.; writing—review and editing, S.Z.; supervision, X.B.; funding acquisition, S.Z., Y.Z. and X.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Natural Science Foundation of China (Grant No. 31960587), the China Agriculture Research System of MOF and MARA (Grant No. CARS-29), the Special Project for Basic Scientific Research of Guangxi Academy of Agricultural Sciences (Grant No. Guinongke2021YT126 and Guinongke2021YT129), and the Project for Science and Technology Development Fund of Guangxi Academy of Agricultural Sciences (Grant No. Guinongke2021JM27).

Data Availability Statement: The data used for the analysis in this study are available within the article and the Supplementary Materials.

Acknowledgments: We thank Metware Biotechnology Co., Ltd. (Wuhan, China) for their support in metabolite detection.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bai, X.; Li, Y.; Huang, J.; Liu, J.; Peng, H.; Xie, T.; Wen, R. One-year-two-harvest cultural technique system for Kyoho grape in southern region of Guangxi. *Southwest China J. Agric. Sci.* **2008**, *21*, 953–955. [CrossRef]
2. Khalil-Ur-Rehman, M.; Wang, W.; Dong, Y.; Faheem, M.; Xu, Y.; Gao, Z.; Guo Shen, Z.; Tao, J. Comparative Transcriptomic and Proteomic Analysis to Deeply Investigate the Role of Hydrogen Cyanamide in Grape Bud Dormancy. *Int. J. Mol. Sci.* **2019**, *20*, 3528. [CrossRef] [PubMed]
3. Guo, R.; Wang, B.; Cheng, G.; Lin, L.; Cao, X.; Zhang, Y.; Xie, T.; Bai, X. Research advances in regionalization for two-crop-a-year grape cultivation in China. *J. South. Agric.* **2016**, *47*, 2091–2097. [CrossRef]
4. Gouot, J.C.; Smith, J.P.; Holzapfel, B.P.; Walker, A.R.; Barril, C. Grape berry flavonoids: A review of their biochemical responses to high and extreme high temperatures. *J. Exp. Bot.* **2019**, *70*, 397–423. [CrossRef]
5. Zhang, C.; Hao, Y.-J. Advances in Genomic, Transcriptomic, and Metabolomic Analyses of Fruit Quality in Fruit Crops. *Hortic. Plant J.* **2020**, *6*, 361–371. [CrossRef]
6. Rienth, M.; Vigneron, N.; Darriet, P.; Sweetman, C.; Burbidge, C.; Bonghi, C.; Walker, R.P.; Famiani, F.; Castellarin, S.D. Grape Berry Secondary Metabolites and Their Modulation by Abiotic Factors in a Climate Change Scenario-A Review. *Front. Plant Sci.* **2021**, *12*, 643258. [CrossRef]
7. Keller, M. *The Science of Grapevines*, 1st ed.; Academic Press: Burlington, MA, USA, 2010; pp. 212–220. [CrossRef]

8. Cheng, G.; He, Y.N.; Yue, T.X.; Wang, J.; Zhang, Z.W. Effects of climatic conditions and soil properties on Cabernet Sauvignon berry growth and anthocyanin profiles. *Molecules* **2014**, *19*, 13683–13703. [CrossRef]
9. Torres, N.; Martinez-Luscher, J.; Porte, E.; Kurtural, S.K. Optimal Ranges and Thresholds of Grape Berry Solar Radiation for Flavonoid Biosynthesis in Warm Climates. *Front. Plant Sci.* **2020**, *11*, 931. [CrossRef]
10. Sun, R.Z.; Cheng, G.; Li, Q.; He, Y.N.; Wang, Y.; Lan, Y.B.; Li, S.Y.; Zhu, Y.R.; Song, W.F.; Zhang, X.; et al. Light-induced Variation in Phenolic Compounds in Cabernet Sauvignon Grapes (Vitis vinifera L.) Involves Extensive Transcriptome Reprogramming of Biosynthetic Enzymes, Transcription Factors, and Phytohormonal Regulators. *Front. Plant Sci.* **2017**, *8*, 547. [CrossRef]
11. Sun, R.Z.; Cheng, G.; Li, Q.; Zhu, Y.R.; Zhang, X.; Wang, Y.; He, Y.N.; Li, S.Y.; He, L.; Chen, W.; et al. Comparative physiological, metabolomic, and transcriptomic analyses reveal developmental stage-dependent effects of cluster bagging on phenolic metabolism in Cabernet Sauvignon grape berries. *BMC Plant Biol.* **2019**, *19*, 583. [CrossRef]
12. Shinomiya, R.; Fujishima, H.; Muramoto, K.; Shiraishi, M. Impact of temperature and sunlight on the skin coloration of the 'Kyoho' table grape. *Sci. Hortic.* **2015**, *193*, 77–83. [CrossRef]
13. Yang, B.; He, S.; Liu, Y.; Liu, B.; Ju, Y.; Kang, D.; Sun, X.; Fang, Y. Transcriptomics integrated with metabolomics reveals the effect of regulated deficit irrigation on anthocyanin biosynthesis in Cabernet Sauvignon grape berries. *Food Chem.* **2020**, *314*, 126170. [CrossRef]
14. Cheng, G.; Zhou, S.; Zhang, J.; Huang, X.; Bai, X.; Xie, T.; Guo, R.; Liu, J.; Yu, H.; Xie, L. Comparison of transcriptional expression patterns of phenols and carotenoids in 'Kyoho' grapes under a two-crop-a-year cultivation system. *PLoS ONE* **2019**, *14*, e0210322. [CrossRef]
15. Cao, X.; Deng, H.; Bai, X.; Zhang, Y.; Wen, R.; Xie, T. Analysis of Anthocyanins in Summer and Winter Fruit of Kyoho Grape by HPLC. *Southwest China J. Agric. Sci.* **2014**, *27*, 1228–1232. [CrossRef]
16. Zhou, Y.; Lin, L.; Wei, R.; Shi, X.; Zhang, Y. Comparative analysis of the quality of three grape varieties in Nanning. *South. Hortic.* **2015**, *26*, 22–24.
17. Cheng, G.; Zhang, J.; Zhou, S.; Xie, L.; Zhang, Y.; Yang, Y.; Guan, J.; Xie, T. Difference in anthocyanin composition between winter and summer grape berries of 'Cabernet Sauvignon' under two-crop-a-year cultivation. *J. Fruit Sci.* **2017**, *34*, 1125–1133. [CrossRef]
18. Chen, W.K. Study of Targeted Metabolome and Transcriptome in Grape Berries Grown under Double Cropping Viticulture System. Ph.D. Thesis, China Agricultural University, Beijing, China, October 2018.
19. Liu, F.Z.; Duan, C.Q. *Technical Manual for Grape Production*, 1st ed.; China Agriculture Press: Beijing, China, 2013; p. 137.
20. Bai, X.; Chen, A.; He, J.; Wang, B.; Qin, B.; Song, Y.; Tang, R. Delayed planting and two-crop-a-year of 'Kyoho' Grape. *South China Fruits* **2016**, *45*, 167–168. [CrossRef]
21. Coombe, B.G. Growth Stages of the Grapevine: Adoption of a system for identifying grapevine growth stages. *Aust. J. Grape Wine Res.* **1995**, *1*, 104–110. [CrossRef]
22. Yu, H.; Li, H.; Wei, R.; Cheng, G.; Zhou, Y.; Liu, J.; Xie, T.; Guo, R.; Zhou, S. Widely Targeted Metabolomics Profiling Reveals the Effect of Powdery Mildew on Wine Grape Varieties with Different Levels of Tolerance to the Disease. *Foods* **2022**, *11*, 2461. [CrossRef]
23. Wang, F.; Huang, Y.; Wu, W.; Zhu, C.; Zhang, R.; Chen, J.; Zeng, J. Metabolomics Analysis of the Peels of Different Colored Citrus Fruits (*Citrus reticulata* cv. 'Shatangju') During the Maturation Period Based on UHPLC-QQQ-MS. *Molecules* **2020**, *25*, 396. [CrossRef]
24. Zou, S.; Wu, J.; Shahid, M.Q.; He, Y.; Lin, S.; Liu, Z.; Yang, X. Identification of key taste components in loquat using widely targeted metabolomics. *Food Chem.* **2020**, *323*, 126822. [CrossRef] [PubMed]
25. Yi, D.; Zhang, H.; Lai, B.; Liu, L.; Pan, X.; Ma, Z.; Wang, Y.; Xie, J.; Shi, S.; Wei, Y. Integrative Analysis of the Coloring Mechanism of Red Longan Pericarp through Metabolome and Transcriptome Analyses. *J. Agric. Food Chem.* **2021**, *69*, 1806–1815. [CrossRef] [PubMed]
26. Xiong, Y.; Yan, P.; Du, K.; Li, M.; Xie, Y.; Gao, P. Nutritional component analyses of kiwifruit in different development stages by metabolomic and transcriptomic approaches. *J. Sci. Food Agric.* **2020**, *100*, 2399–2409. [CrossRef] [PubMed]
27. Sun, T.; Zhang, J.; Zhang, Q.; Li, X.; Li, M.; Yang, Y.; Zhou, J.; Wei, Q.; Zhou, B. Integrative physiological, transcriptome, and metabolome analysis reveals the effects of nitrogen sufficiency and deficiency conditions in apple leaves and roots. *Environ. Exp. Bot.* **2021**, *192*, 104633. [CrossRef]
28. Peng, L.; Gao, W.; Song, M.; Li, M.; He, D.; Wang, Z. Integrated Metabolome and Transcriptome Analysis of Fruit Flavor and Carotenoids Biosynthesis Differences Between Mature-Green and Tree-Ripe of cv. "Golden Phoenix" Mangoes (*Mangifera indica* L.). *Front. Plant Sci.* **2022**, *13*, 816492. [CrossRef]
29. Wang, B.; He, J.; Duan, C.; Yu, X.; Zhu, L.; Xie, Z.; Zhang, C.; Xu, W.; Wang, S. Root restriction affects anthocyanin accumulation and composition in berry skin of 'Kyoho' grape (*Vitis vinifera* L. × *Vitis labrusca* L.) during ripening. *Sci. Hortic.* **2012**, *137*, 20–28. [CrossRef]
30. Flamini, R.; Mattivi, F.; De Rosso, M.; Arapitsas, P.; Bavaresco, L. Advanced knowledge of three important classes of grape phenolics: Anthocyanins, stilbenes and flavonols. *Int. J. Mol. Sci.* **2013**, *14*, 19651–19669. [CrossRef]
31. Zhu, S.; Liang, Y.; An, X.; Kong, F.; Yin, H. Response of fruit quality of table grape (*Vitis vinifera* L.) to foliar selenium fertilizer under different cultivation microclimates. *Eur. J. Hortic. Sci.* **2019**, *84*, 332–342. [CrossRef]

32. Wang, W.; Khalil-Ur-Rehman, M.; Wei, L.L.; Nieuwenhuizen, N.J.; Zheng, H.; Tao, J.M. Effect of Thidiazuron on Terpene Volatile Constituents and Terpenoid Biosynthesis Pathway Gene Expression of Shine Muscat (*Vitis labrusca* x *V. vinifera*) Grape Berries. *Molecules* **2020**, *25*, 2578. [CrossRef]
33. Xue, H.K.; Tan, J.Q.; Liu, C.; Liu, C.H. Isolation, Purification, Structure Identification and Anti-tumor Activity of Anthocyanin from Kyoho Grape Skins. *Food Sci.* **2020**, *41*, 39–48. [CrossRef]
34. Liao, H.S.; Yang, C.C.; Ming-Hsiun, H. Nitrogen deficiency- and sucrose-induced anthocyanin biosynthesis is modulated by histone deacetylase15 in arabidopsis. *J. Exp. Bot.* **2022**, *11*, 3726–3742. [CrossRef]
35. Poudel, P.R.; Koyama, K.; Goto-Yamamoto, N. Evaluating the influence of temperature on proanthocyanidin biosynthesis in developing grape berries (Vitis vinifera L.). *Mol. Biol. Rep.* **2020**, *47*, 3501–3510. [CrossRef]
36. Azuma, A.; Yakushiji, H.; Koshita, Y.; Kobayashi, S. Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions. *Planta* **2012**, *236*, 1067–1080. [CrossRef]
37. Yin, Y.; Li, M.; Jia, N.; Sun, Y.; Han, B.; Liu, C.; Liu, S.; Zhao, S.; Guo, Z. Effects of trellis system and berry thinning intensity on vine performance and quality composition of two table grape cultivars under protected cultivation in northern China. *Sci. Hortic.* **2022**, *299*, 111045. [CrossRef]
38. Carbonell-Bejerano, P.; Diago, M.-P.; Martínez-Abaigar, J.; Martínez-Zapater, J.M.; Tardáguila, J.; Núñez-Olivera, E. Solar ultraviolet radiation is necessary to enhance grapevine fruit ripening transcriptional and phenolic responses. *BMC Plant Biol.* **2014**, *14*, 183. [CrossRef]
39. Zhang, K.; Li, W.; Ju, Y.; Wang, X.; Sun, X.; Fang, Y.; Chen, K. Transcriptomic and Metabolomic Basis of Short- and Long-Term Post-Harvest UV-C Application in Regulating Grape Berry Quality Development. *Foods* **2021**, *10*, 625. [CrossRef]