



## Nutrient composition and functional constituents of daylily from different producing areas based on widely targeted metabolomics

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### ABSTRACT

Daylily is a functional food with high nutritional value in China. Datong (DT) in Shanxi Province is one of the four main production areas of daylily. Therefore, Linfen (LF), Lvliang (LL), and Yangquan (YQ) in Shanxi Province have also introduced daylily from DT. However, geographical and climatic conditions and producing patterns cause variations in the daylily quality. In the present study, we found that the nutrient composition of daylilies from different producing areas of Shanxi Province varied. The key environmental factors affecting the nutrition of daylily in different regions were altitude and temperature. The widely targeted metabolomics results showed that 1642 metabolites were found in daylily. The differential metabolites between DT and YQ, LL and LF were 557, 667, and 359, respectively. Notably, 9 metabolic pathways and 59 metabolite markers were associated with daylily from different areas. This study provides a theoretical basis for the quality maintenance and health efficacy research of daylily.

### 1. Introduction

*Hemerocallis citrina Baroni* (Daylily), belonging to the family of Asphodelaceae, is a species of herbaceous perennial plant. As a kind of vegetable that people in Asia widely consume, the daylily is cultivated in China, Japan, and Korea due to its high adaptability to different environmental conditions and abundant germplasm resources, and its flower buds are the major edible part (Ou, Liu, & Wu, 2020). In China, Shanxi province has distinct four seasons, synchronous rain and heat, sufficient sunlight, and a significant day/night temperature difference, making it suitable for growing daylily. In particular, Datong (DT) in Shanxi is one of the main daylily production areas in China. Most of the daylilies in DT grows near the Datong Volcanic Group; volcanic soil is rich in essential nutrients that stimulate plant growth. In addition, DT has a high local temperature difference between day and night, with a noticeable temperature difference of 20°C between day and night (Li, Cui, Qin, Wang, & Wang, 2023). Therefore, the daylily in DT is of higher quality. Moreover, daylily has been a healthy food for at least 400 years. Liang et al., (Liang et al., 2023) demonstrated the sleep-improving effects of

daylily. The water and ethanol extracts of daylily flowers had significant anti-depressive activities, with water extract having the most robust function compared to other extracts (Liu et al., 2022). Rutin, one of the primary active compounds in the water extract of daylily, showed significant antidepressant activity. Furthermore, our previous study found that daylily buds can ameliorate the insufficient lactation of rats, and the active components promoting lactation may be flavonoids and phenols (Guo et al., 2023). Therefore, the daylily has received widespread attention due to its rich nutritional functions, and many regions (i.e., Linfen (LF), Lvliang (LL), and Yangquan (YQ)) in Shanxi Province have also introduced daylily in DT for cultivation.

However, different growing environments significantly affect the quality of plants (vegetables or herbs) (Gao et al., 2021). In particular, the average annual temperature in different regions of Shanxi Province increases from the north to the south and decreases from the basins to the high mountains (Xu et al., 2020). Precipitation is also distributed differently across the Shanxi province, depending on topography. Also, the various environmental conditions, such as temperature, light intensity, precipitation, and soil in different regions of Shanxi Province,

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may lead to differences in the nutritional quality of daylily. Determining the nutritional quality of daylily in different producing areas will contribute to its development as a functional food. The metabolomic analysis offers a comprehensive analysis of chemical components for characterizing food quality and could identify thousands of metabolites in terms of quality and quantity (Li et al., 2022).

This study adopted widely targeted metabolomics to qualitatively and quantitatively evaluate the nutrient composition and functional constituents of daylily from four producing areas in Shanxi Province. In addition, the relationship between nutrient composition, functional constituents and environmental factors were also investigated. It may help identify the characteristic constituents of daylily and promote its widespread processing and utilization.

## 2. Materials and methods

### 2.1. Preparation of daylily samples

Daylily samples were collected on July 2022 from four plant areas, including DT (113°32' 27.75" N, 40° 05' 24.32" E), LF (112°02' 17.6" N, 36° 13' 13.15" E), YQ (113°38' 24.94" N, 37° 59' 11.61" E) and LL (111°16' 31.79" N, 37° 56' 09.99" E) in Shanxi Province (Fig. 1). Based on the color, shape, and size of the daylily, we determined whether it

was ripe. The flower buds of the ripe daylily from four daylily-producing areas were picked between 6 and 7 am and immediately frozen in liquid nitrogen for metabolomics analysis. The daylily samples were vacuum freeze-dried and ground (30 Hz, 1.5 min) into powder using a grinder for nutrients determination.

### 2.2. Determination of environmental factors

The soil alkali-hydro nitrogen content was determined using the alkaline diffusion method according to the Chinese industry standard: LY/T 1228–2015; the soil available phosphorus content was determined using sodium bicarbonate extraction and molybdenum antimony colorimetric method according to Chinese industry standard: NY/T 1121.7–2014; the available potassium content in soil was measured using ammonium acetate extraction method combined with flame photometry according to Chinese industry standard: NY/T 889–2004. In addition, soil available selenium content was determined using hydride generation atomic fluorescence spectroscopy. The soil organic matter content was determined using potassium dichromate oxidation combined with the external heating method according to Chinese industry standard NY/T 1121.6–2006. The soil pH was measured by a pH potentiometer.

Climate conditions in different daylily-producing areas of Shanxi

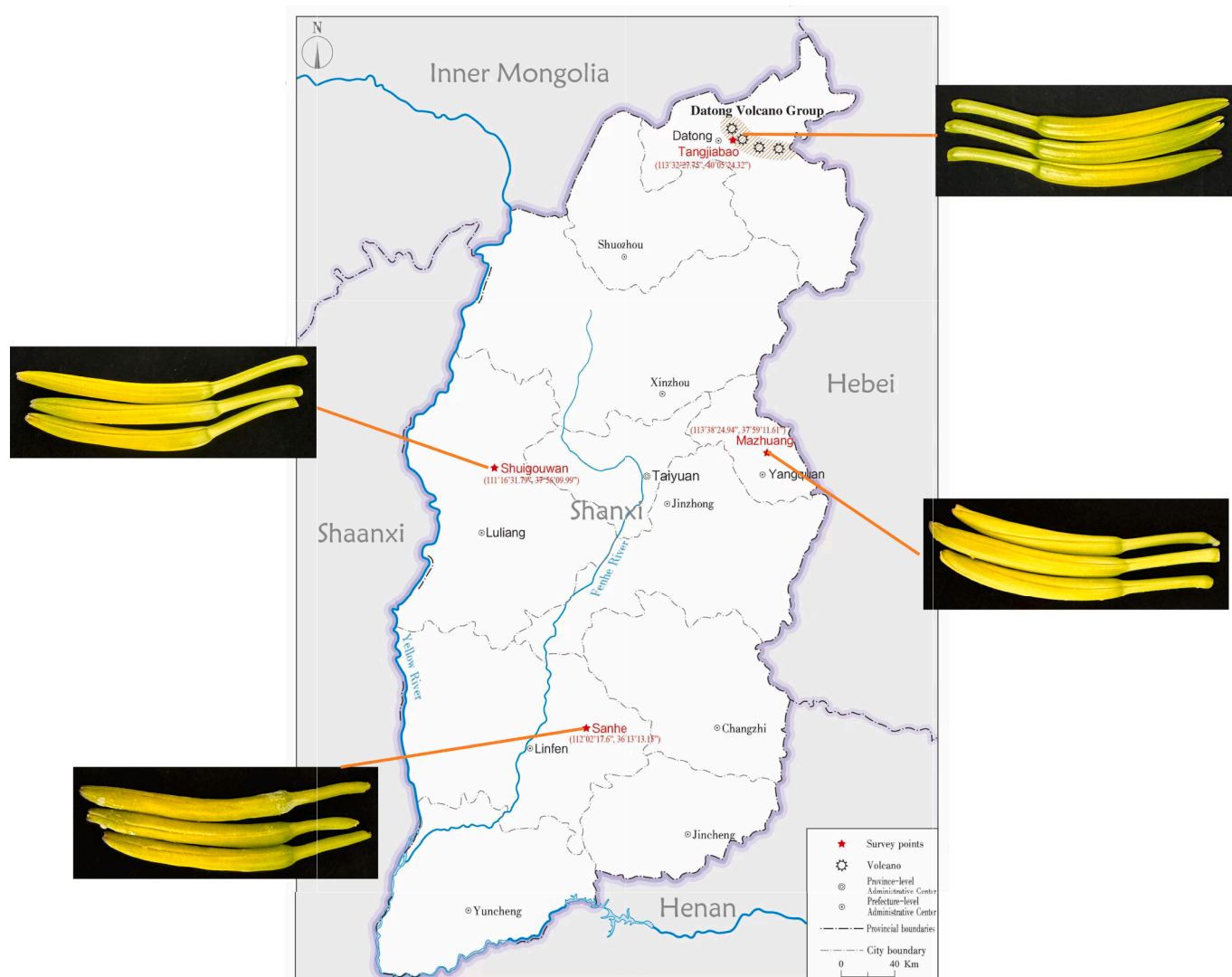


Fig. 1. Geographical distribution of four producing areas of daylily in Shanxi Province.

Province can be downloaded on the website of China meteorological data (<http://data.cma.cn/>), including daily average temperature, daily maximum temperature, daily minimum temperature, relative humidity, sunlight hours, and annual precipitation. In addition, the altitude information was obtained through real-time positioning using Ovi interactive map software during sampling in different areas. Detailed climate factors for each sampling site are listed in [Table S1](#).

### 2.3. Proximate composition analysis

The moisture content was measured using the direct drying method in GB/T 5009.3–2016. Amino acid content was measured using an amino acid analyzer based on the acid hydrolysis method. The protein content in daylily samples was analyzed using the Kjeldahl nitrogen method.

### 2.4. The determination of total flavonoid content (TFC) and total polyphenol content (TPC)

**TFC:** 0.25 g of daylily sample was mixed with 5 ml of 70 % ethanol (v/v) and reacted for 30 min under ultrasonic treatment (60°C, 80W). The process was repeated twice. The supernatant extracted twice was merged to form the daylily flavonoid extraction solution, and the total volume was recorded as  $V_1$ . After dissolving in 70 % ethanol, the rutin standard was diluted to different mass concentrations of 0.500 mg/ml, 0.250 mg/ml, 0.125 mg/ml, 0.063 mg/ml, and 0.031 mg/ml. Then 20  $\mu$ L of  $\text{NaNO}_2$  was added to 50  $\mu$ L of rutin or sample solution and allowed to stand for 5 min, then 20  $\mu$ L of 10 %  $\text{AlCl}_3$  solution was added and allowed to stand for 6 min, and finally 200  $\mu$ L of 4 % NaOH solution was added and allowed to stand for 15 min. The absorbance at 510 nm was measured. The concentration of flavonoids (mg/ml) in reaction solution was calculated according to a standard curve with the concentration of rutin as the x-axis and the absorbance as the y-axis. The TFC was calculated based on the following formula.

$$\text{TFC (mg/g)} = V_1 \times C_1 \times D_1 / M_1$$

$V_1$ - volume of the test solution (mL);  $C_1$ - the concentration of flavonoid (mg/mL) in reaction solution obtained from the standard curve;  $D_1$ - dilution ratio of polyphenol extraction solution;  $M_1$ - sample mass (g).

**TPC:** Weigh 0.25 g of daylily powder, add 3.75 ml of 60 % ethanol solution, and ultrasonic extraction was performed at 50 °C and 200 W for 70 min. The resultant solution was centrifuged (4°C, 6000 rpm, 10 min). The process was repeated twice. The supernatant extracted twice was merged to form the daylily polyphenol extraction solution, and the total volume was recorded as  $V_1$ . Then, 10 mg of the gallic acid standard was dissolved in distilled water to obtain standard solutions (0.05, 0.10, 0.15, 0.20, and 0.25 mg/ml). 1.0 ml of the standard solution or sample solution was placed in a colorimetric tube, 5.0 ml of distilled water and 2.0 ml of Folin-Ciocalteu reagent were added, and reacted for 4 min in the dark. 2.0 ml of 10 %  $\text{Na}_2\text{CO}_3$  solution was added to form solutions of 5, 10, 15, 20, 25  $\mu$ g/mL concentration, and reacted in a water bath in the dark (30 °C, 30 min). The absorbance was measured using a UV spectrophotometer at 765 nm, and the standard curve was plotted with the concentration of gallic acid as the x-axis and the absorbance as the y-axis, the concentration of polyphenols (mg/ml) in reaction solution was obtained from the standard curve. The TPC was calculated based on the following formula and a standard curve was plotted.

$$\text{TPC (mg/g)} = \frac{V_1 \times C_1 \times D_1}{M_1}$$

$V_1$ - volume of the test solution (mL);  $C_1$ - the concentration of polyphenols (mg/mL) in reaction solution obtained from the standard curve;  $D_1$ - dilution ratio of polyphenol extraction solution;  $M_1$ - sample mass (g).

### 2.5. The preparation and extraction of daylily sample

50 mg of sample powder was weighed using an electronic balance, Add 1200  $\mu$ L of  $-20$  °C pre-cooled 70 % methanolic aqueous internal standard extract was added, and vortex once every 30 min for 30 s, with six vortices. After centrifugation at 12000 rpm for 3 min, the supernatant was collected. It was then filtered through a microporous membrane with a pore size of 0.22  $\mu$ m for UPLC-MS/MS analysis.

### 2.6. Widely targeted metabolomics analysis

The samples were analyzed by a UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD, <https://sciex.com.cn/>; MS, Applied Biosystems 6500 Q TRAP, <https://sciex.com.cn/>). The separation was performed on an Agilent SB-C18 column (2.1 mm\*100 mm) and the flow rate was 0.35 ml per minute. The mobile phase A was pure water containing 0.1 % formate; and the mobile phase B was acetone containing 0.1 % formate. The gradient program was used to measure samples, and the initial conditions were 95 % A and 5 % B. The linear gradient to 5 % A and 95 % B was programmed within 9 min, and the components of 5 % A and 95 % B were kept for 1 min. This was followed within 1.1 min by an adjustment to 95 % A, 5.0 % B and kept for 2.9 min. The injection volume was 2  $\mu$ L.

The operating parameters of the ESI source were: source temperature 500 °C; ion spray voltage (IS) 5,500 V (positive ion mode)/4,500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set to 50, 60 and 25 psi, respectively; collision-activated dissociation (CAD) was high. QQQ experiments were performed by multiple reaction monitoring (MRM) using nitrogen as the impingement gas. The declustering potential (DP) and collision energy (CE) for individual MRM transitions were carried out with further optimization of the DP and CE. For each time period, according to the metabolites eluted within that time period, a specific set of MRM transitions was monitored.

### 2.7. Multivariate statistical analysis, kyoto encyclopedia of genes and genomes (KEGG) annotations and metabolic pathway analyses of differential metabolites

The unsupervised principal component analysis (PCA) was performed by R packages (<https://www.r-project.org>). Hierarchical cluster analysis (HCA) results of metabolites in all samples were shown as heatmaps with dendrograms. They were performed using the R package ComplexHeatmap. VIP (VIP > 1) and absolute Log2Fold change (FC) ( $|\text{Log}_2\text{FC}| \geq 1.0$ ) were used to determine differential metabolites for the two-group analysis. The R package MetaboAnalystR extracted VIP values from the orthogonal partial least squares discriminate analysis (OPLS-DA) result. The data were log-transformed and meant-centered before OPLS-DA. A permutation test (200 permutations) was performed to avoid overfitting.

The KEGG Compound database (<https://www.kegg.jp/kegg/compound/>) was used for metabolite annotation, and the annotations were then mapped to the KEGG Pathway database (<https://www.kegg.jp/kegg/pathway.html>). Significantly regulated pathways mapped by the metabolites were then subjected to metabolite set enrichment analysis (MSEA), with significance determined by the p-values of the hypergeometric test.

### 2.8. Data analysis

The data are expressed as mean values  $\pm$  standard deviation (SD). The statistical significance of  $p < .05$  was determined by ANOVA and Duncan multiple comparison. SPSS 22.0 software (IBM Corporation, Chicago, IL, USA) was adopted for statistical calculations and analyses. Redundancy analysis (RDA) was conducted in Canoco 5.0 to determine the relationship between nutritional components in daylily and environmental factors. According to RDA, the contribution of the

environmental factors to nutritional components in daylily was evaluated by the software and showed in the x and y axes. The Pearson correlation analysis method was used to analyze the relationship between metabolites and environmental factors in different daylily-producing regions.

### 3. Results and discussion

#### 3.1. Environmental factors analysis

Table S1 shows the climate factors of different daylily-producing areas in Shanxi. The daily average, maximum, and minimum temperatures in the LL were the highest, followed by the YQ, LF, and DT. LL also has the highest value for relative humidity, followed by LF, YQ, and DT. In addition, the ranking of annual rainfall was LF, YQ, LL, and DT. Also, DT had the longest sunlight hours, while LL had the shortest. Moreover, LL had the highest altitude, followed by DT, LF, and YQ. Overall, there was significant differences between soil nutrients and climate factors in the four daylily-producing areas. Table S2 shows the soil nutrients of different daylily-producing areas in Shanxi. The organic matter and available phosphorus content in LL were the highest, followed by YQ, DT, and LF. The available potassium content in the LL was also the highest, followed by the DT, YQ, and LF. YQ had the highest content for available selenium, and LF had the lowest content. In addition, the ranking of alkali-hydro nitrogen content was DT > LL > YQ > LF. The pH ranking was LF > DT > LL > YQ.

#### 3.2. The proximate composition, amino acid, TFC and TPC in daylily of different producing areas

The proximate composition, amino acid, TFC, and TPC were determined to assess the variation in the nutritional profile of daylily in different growing areas (Table S3 and Table 1). Compared to DT, the protein content in YQ was significantly increased. There was no significant difference of protein content present between the DT, LF and LL. The contents of total amino acids (TAA) and essential amino acids (EAA) in the DT were significantly lower than in YQ, LL and LF. Food proteins generally comprise  $\alpha$ -amino acids and the taste of  $\alpha$ -amino acids is classified as: Glu and Asp have an umami taste; Gly and Ala are mainly sweet (Reiko, Rio, Yuna, & Masataka, 2022). The umami taste amino acids (UTAA) were highest in YQ, followed by LF, DT, and LL. While the order of content of sweet amino acid (SAA) content was YQ > LF > LL > DT. The moisture content of YQ, LL, and LF was lower than that of DT. Moreover, apparent differences of TPC and TFC were also observed between the DT and LL, YQ, LF. The highest TFC was observed in LL (71.19 mg RE/g), followed by DT (52.02 mg RE/g), LF (47.24 mg RE/g), and YQ (46.0 mg RE/g). While the highest TPC was present in DT (13.96 mg GAE/g), followed by LF (10.01 GAE/g), LL (9.70 mg GAE/g), and YQ (8.23 GAE/g). Overall, the nutrient composition of daylilies from different producing areas varied.

#### 3.3. The relationship between environmental factors and nutritional components in daylily of different producing areas

RDA was used to evaluate the relationship between the environmental factors and nutritional components in daylily (Fig. S1). The environmental factors affecting the nutritional differences of daylily in different regions were identified as altitude, daily average temperature, daily maximum temperature, organic matter, available selenium, and available potassium, accounting for 79.2 %, 15.3 %, 3.9 %, 0.9 %, 0.4 % and 0.1 % of the distribution of nutritional components in daylily. Each typical axis mentioned above was significant ( $p < 0.05$ ), indicating that the interpretation of environmental factors (altitude, daily average temperature, daily maximum temperature, organic matter, available selenium, and available potassium) was credible. The key environmental factors affecting the nutrition of daylily in different regions were

**Table 1**

The amino acid composition of daylily in different producing areas (unit: g/100 g daylily sample).

	DT	YQ	LL	LF
Asp	0.900 ± 0.002b	1.219 ± 0.010a	0.258 ± 0.002d	0.660 ± 0.001c
Thr	0.004 ± 0.000d	0.647 ± 0.005a	0.148 ± 0.000c	0.376 ± 0.013b
Ser	0.087 ± 0.009c	0.251 ± 0.001b	0.370 ± 0.002ab	0.404 ± 0.013a
Glu	0.400 ± 0.000d	3.005 ± 0.010a	0.591 ± 0.004c	1.833 ± 0.059b
Gly	0.128 ± 0.012c	0.694 ± 0.006a	0.180 ± 0.035c	0.352 ± 0.012b
Ala	0.196 ± 0.006d	0.570 ± 0.001b	0.270 ± 0.003c	0.640 ± 0.018a
Cys	—	—	—	—
Val	0.157 ± 0.011c	0.723 ± 0.002a	0.160 ± 0.001c	0.368 ± 0.009b
Met	0.035 ± 0.002d	0.153 ± 0.003a	0.050 ± 0.001c	0.070 ± 0.002b
Ile	0.134 ± 0.010d	0.552 ± 0.000a	0.190 ± 0.006c	0.298 ± 0.009b
Leu	0.218 ± 0.019d	0.860 ± 0.001a	0.280 ± 0.001c	0.475 ± 0.018b
Tyr	0.098 ± 0.005c	0.309 ± 0.003a	0.080 ± 0.016c	0.191 ± 0.007b
Phe	0.129 ± 0.012c	0.487 ± 0.003a	0.122 ± 0.016c	0.275 ± 0.008b
His	0.038 ± 0.001d	0.070 ± 0.001c	0.215 ± 0.007b	0.523 ± 0.011a
Lys	0.150 ± 0.004c	0.649 ± 0.001a	0.132 ± 0.009c	0.578 ± 0.002b
Arg	0.139 ± 0.008d	0.544 ± 0.018a	0.187 ± 0.009c	0.387 ± 0.012b
Pro	0.124 ± 0.010c	0.269 ± 0.021a	0.174 ± 0.003b	0.253 ± 0.000a
Total amino acids	2.804 ± 0.269d	11.001 ± 0.042a	3.408 ± 0.060c	7.508 ± 0.357b
Essential amino acids	0.827 ± 0.057d	4.070 ± 0.004a	1.083 ± 0.022c	2.439 ± 0.052b
Umami taste amino acids	1.300 ± 0.002c	4.224 ± 0.018a	0.848 ± 0.005d	2.494 ± 0.057b
Sweet amino acids	0.324 ± 0.017d	1.263 ± 0.006a	0.450 ± 0.037c	0.992 ± 0.030b

altitude and temperature.

We further analyzed the relationship between nutritional components and environmental factors in daylily from four regions in Shanxi Province (Fig. S1). Amino acids, flavonoids, and phenolic acids are abundant in both medicinal plants and plants used for food, and play a significant role in our everyday lives (Han et al., 2023). Amino acids play an important role in plant response to pathogenic stress (Rai, 2002). The protein, TAA, SAA, EAA, and UTAA contents were positively related to daily average temperature, daily maximum temperature, organic matter, and available selenium and negatively related to altitude and available potassium. In the present study, daylily in YQ had the highest protein content, TAA, EAA, SAA, and UTAA, which could be related to its lowest altitude and highest available selenium content. Meanwhile, the lowest levels of protein, TAA, EAA, SAA, and UTAA in the daylily of the DT were correlated with its lowest daily average and maximum temperatures. Therefore, daylily in YQ can better cope with pathogenic stress due to its high content of amino acids. In addition, phenolic compounds are being used by the food, pharmaceutical, health, and cosmetic industries (Babuskin et al., 2014). Phenolics have an important role in plant metabolism. They accumulate in plants in response to stressful conditions, such as harmful environmental circumstances (drought, extreme temperatures, pollution, etc.) (Samec, Karalija, Sola, Vujcic, & Salopek-Sondi, 2021). The TPC was positively related to available potassium and altitude and negatively related to daily average and maximum temperatures, available selenium, and organic matter. While the highest TPC in DT was related to its lowest daily average

temperature and maximum temperature. Thus, daylily in DT can better resist harmful environmental conditions. While flavonoids, as naturally occurring substances in plants, can be attributed to the family of coloring agents and play a significant role as pigments. They can also be used as antioxidants to fight against free radicals, including flavonoids, isoflavones, flavonoids, anthocyanins, and catechins (Sim & Han, 2008). The TFC was positively related to altitude, daily average and maximum temperatures, available potassium and organic matter, and negatively to available selenium. The highest altitude, average and maximum temperatures, soil available potassium, and organic matter in LL contributed to its highest TFC. Therefore, daylily in the LL may function well in clearing free radicals. Overall, low altitude and high temperature, and soil rich in organic matter and available selenium are beneficial for cultivating daylily with high amino acid and protein contents; high altitude, low temperature, and abundant available potassium in soil are conducive for the enrichment of TPC in daylily; high temperature and altitude, and soil rich in available potassium and organic matter are effective in enriching TFC in daylily.

### 3.4. Overview of the metabolites identified in daylily

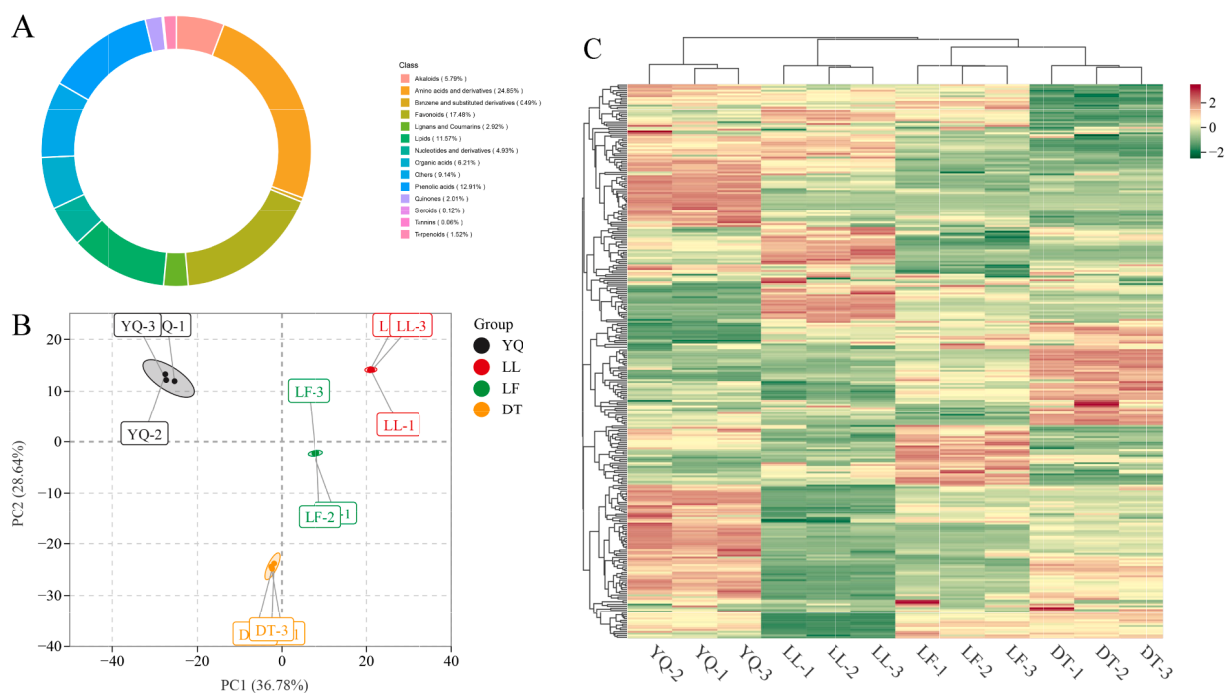
A total of 1642 metabolites were identified in daylily (Table S4), including 5.79 % alkaloids, 24.85 % amino acids and derivatives, 0.49 % benzene and substituted derivatives, 17.48 % flavonoids, 2.92 % lignans and coumarins, 11.57 % lipids, 4.93 % nucleotides and derivatives, 6.21 % organic acids, 9.14 % others, 12.91 % phenolic acids, 2.01 % quinones, 0.12 % steroids, 0.06 % tannins and 1.52 % terpenoids (Fig. 2A), more than in a previous study where 641 and 648 compounds were found in freeze-dried and oven-dried daylily, respectively (Guo et al., 2023). This study identified 408 amino acids and their derivatives, the highest proportion of metabolites. Many amino acids and their derivatives, even those that have no role in protein synthesis, are essential for plant development and responses to environmental stresses (Cai & Aharoni, 2022). In addition, amino acids are the starting point of

numerous primary and secondary metabolic products and are critical in human health benefits (Trovato, Funck, Forlani, Okumoto, & Amir, 2021). Moreover, amino acids are important components that determine the flavor of daylily (Li-Chan & Cheung, 2010).

Flavonoids were the second abundant compounds in daylily. Many flavonoids, for example, quercetin, have biological activities that can contribute to human health (Zymone, Benetis, Trumbeckas, Baseviciene, & Trumbeckaite, 2022). The third most abundant metabolite identified in daylily was phenolic acids. In our study, 287 flavonoids were identified, among which 1 was dihydroisoflavones, 8 were flavanols, 17 were chalcones, 30 were flavanones, 7 were flavanonols, 79 were flavones, 121 were flavonols, 8 were isoflavones, and 16 were other flavonoids. Previous studies have shown that flavonoids make up about two-thirds of dietary phenolics. There is increasing awareness and interest in the antioxidant activity and possible health benefits of phenolic acids, which account for almost all of the remaining third (Shahrajabian, 2019). Also, 212 phenolic acids were detected in daylily. In addition to being antioxidants, these phenolic acids exhibited various physiological effects.

Lipids play many important physiological roles, such as producing membranes, storing energy, and forming signal molecules (Huang et al., 2022). Nevertheless, little is known about the lipids found in daylilies. In the present study, we found 190 lipids, thus filling in the blanks in our knowledge of daylily lipids. Among them, the lipids in daylily were mainly free fatty acids (FAAs) and lysophosphatidyl choline (LPC). Besides essential energy sources, FAAs act as signal molecules regulating various cellular processes and physiological functions depending on carbon chain length (Miyamoto et al., 2016). On the other hand, LPC is a vital signal molecule with diverse biological functions that regulate cellular inflammation (Zhang et al., 2018). Therefore, the high content of FAAs and LPC in daylily makes it a food with good nutritional value.

In addition, we identified 95 alkaloids, 8 benzene, and substituted derivatives, 48 lignans and coumarins, 81 nucleotides and derivatives, 102 organic acids, 150 others, 33 quinones, 25 terpenoids, 2 steroids, 1



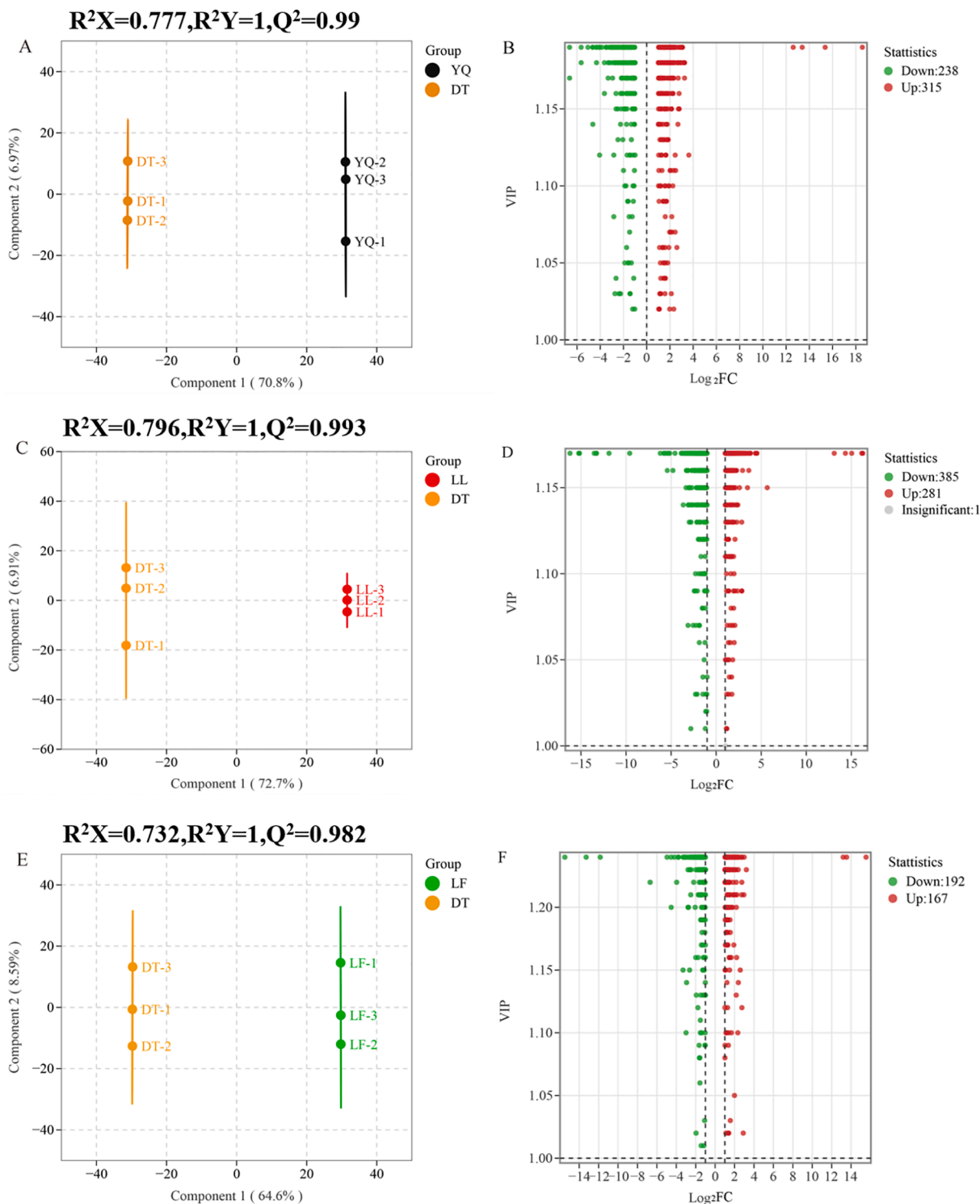
**Fig. 2.** A: Classification of the 1642 metabolites in daylily samples; B: PCA score plot; C: HCA results of all metabolites in all samples. Each sample was represented by a column, and each metabolite was represented by a row. The abundance of each metabolite was represented by a bar with a specific color. The up-regulated and down-regulated metabolites were indicated by different shades of red and green, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tannin in daylily. It is worth noting that, although there were differences among DT, YQ, LL, and LF groups, their metabolite distributions were the same.

### 3.5. Identification of differential metabolites

Daylily from four regions of Shanxi Province were collected to determine the differences in nutritional components. By performing PCA

analysis on the samples, we can determine the overall metabolite differences and the degree of variability between samples in different groups (Chen, Martin, & Montague, 2009). As shown in Fig. 2B, the grouping ellipses of metabolites in daylily from four producing areas were separated, and the total interpretation rate was 65.42% (PC1 + PC2). Furthermore, HCA was conducted based on the content of metabolites. The results showed that the metabolic spectrum of daylily in DT and LF was the closest, followed by LL and YQ (Fig. 2C).



**Fig. 3.** The OPLS-DA score plots of daylily obtained from four producing areas in Shanxi Province. A, C, E, OPLS-DA model plots for the comparison groups DT vs. YQ, DT vs. LL, DT vs. LL, respectively; B, D, F: The volcano plots that present the number of differentially metabolites in DT vs. YQ, DT vs. LL, DT vs. LF, respectively.

In addition, we performed OPLS-DA to screen the metabolites that correspond to the differences among these four groups (Fig. 3). OPLS-DA can preliminarily screen differential metabolites between different cultivars or tissues. Parameters, including R2X, R2Y, and Q2, evaluated the OPLS-DA model. Specifically, the R2X and R2Y indicate the explanatory power of the constructed OPLS-DA model to the X and Y matrices, respectively, while Q2 refers to the model's predictive power. The closer R2X and R2Y are to 1, the more stable and reliable the model is. When  $Q2 > 0.5$ , it can be considered a valid model; if  $Q2 > 0.9$ , it is an excellent model. In our study, the Q2 values of the four comparison groups were above 0.9, suggesting that the OPLS-DA models were stable. The OPLS-DA plots exhibited that the daylily from four producing areas were separated, indicating significant differences among these four groups. The R2X, R2Y, and Q2 for DT and YQ were 0.777, 1, 0.99; DT and LL were 0.796, 1, 0.993; DT and LF were 0.732, 1, 0.982 (Fig. 3A, C, E). Collectively, the metabolic profile of daylily in DT was distinct from that in YQ, LL, and LF.

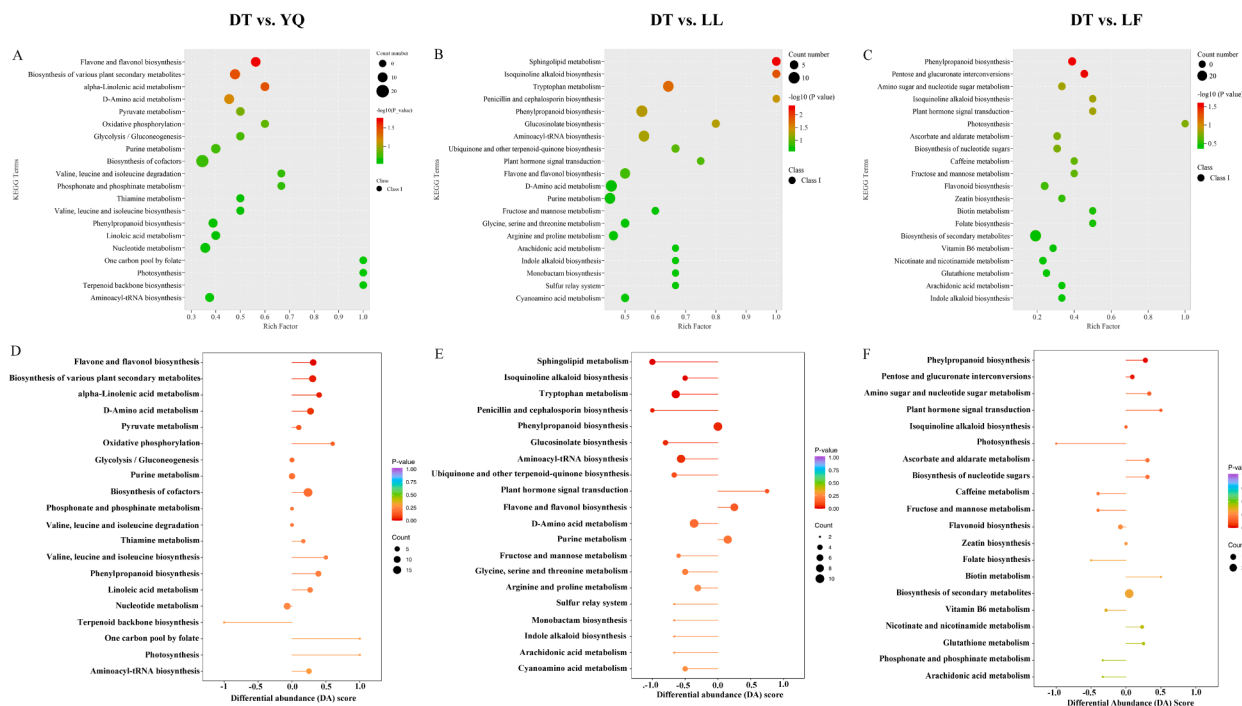
A total of 557 differential metabolites between DT and YQ were identified ( $FC > 1$  or  $< 0.5$  and  $VIP > 1$ ), among which 315 metabolites were up-regulated, and 238 metabolites were down-regulated. In addition, there were 667 differential metabolites between DT and LL (281 up-regulated, 385 down-regulated). Also, 359 differential metabolites were detected between DT and LF (167 up-regulated, 192 down-regulated). In particular, the highest proportion of these differential metabolites was amino acids and derivatives, flavonoids and phenolic acids (Fig. 3B, D, F). Venn analysis can not only display the number of metabolites in each group, but also present the overlapping relationship of metabolites among different metabolic groups. As shown in Fig. S2, the number of all metabolites in the four comparison groups was different. The unique number of metabolites in the DT and YQ metabolite sets, DT and LL metabolite sets, and DT and LF metabolite sets was 184, 243, and 59, respectively. There were a total of 141 metabolites in the three metabolomics, including 8 alkaloids, 45 amino acids and derivatives, 2 benzene and substituted derivatives, 28 flavonoids, 5 lignans

and coumarins, 6 lipids, 2 nucleotides and derivatives, 9 organic acids, 3 others, 28 phenolic acids, 2 quinones, 3 terpenoids (Table S5). In addition, we investigated the proportion of nutrient content, as well as nutrient content of metabolites in daylily from different growing areas. The flavonol content in daylily from DT and YQ were the most different, indicating that the climatic conditions in YQ were conducive to flavonol accumulation (Fig. S3A-B). The contents of amino acids and derivatives, and LPC in daylily of DT and LL were the most different, suggesting that the DT climate was conducive to the formation of amino acids and derivatives (Fig. S3C-D). The differences between the DT and LF groups were relatively small, with significant changes in the content of amino acids, derivatives, and flavonoids (Fig. S3E-F).

### 3.6. KEGG annotation and enrichment analysis

#### 3.6.1. Metabolite differences between DT vs. YQ

In this research, we identified 557 metabolites between DT and YQ groups, including 29 alkaloids, 152 amino acids and derivatives, 5 benzene and substituted derivatives, 114 flavonoids, 15 lignans and coumarins, 56 lipids, 25 nucleotides and derivatives, 25 organic acids, 32 others, 79 phenolic acids, 14 quinones, 10 terpenoids, 1 tannin (Table S6). Further analysis was conducted on the critical metabolic pathways and metabolite levels in the DT and YQ comparison group. The smaller the p-value of the differential metabolic pathway, the more significant the pathway enrichment. The flavone and flavonol biosynthesis, biosynthesis of various plant secondary metabolites, and  $\alpha$ -linolenic acid metabolism pathway were three pathways that corresponded to the differential metabolites of DT and YQ groups, including 9, 11, and 6 metabolites, respectively (Fig. 4A-C, Table S9), with a total of 14 metabolites increased and 12 metabolites decreased. The difference in abundance (DA) scores could reflect the overall changes in all pathways. A DA score of 1 indicates an up-trend in the expression of all identified pathways, and a DA score of  $-1$  suggests a down-trend. The absolute value of the DA score is presented by the length of the line, and the size



**Fig. 4.** A-C, KEGG enrichment of differential metabolites between the comparison groups (DT vs. YQ/LL/LF). Each bubble in the plot represents a metabolic pathway whose abscissa and bubble size together indicate the magnitude of the pathway's impact factors. A larger bubble size indicates a larger impact factor. The bubble colors represent the p-values of the enrichment analysis, with redder colors indicating a higher degree of enrichment. D-F: Differential abundance (DA) score of metabolic pathways between the different comparison groups (DT vs. YQ/LL/LF).

of the dot at the end of the liners corresponds to the number of differentially expressed metabolites in the pathways. Dot distribution is left of the axis and longer lines indicate a tendency for the downregulation of general pathway expression. If dots are distributed to the right of the axis, longer lines indicate that overall pathway expression tends to increase. The more metabolites there are, the more prominent the dots. The size of the p-value is displayed by the color of the line segments and points. The more it tends to red, the smaller the p-value, and the more it tends to purple, the larger the p-value. In the present study, the three identified pathways, including flavone and flavonol biosynthesis, biosynthesis of various plant secondary metabolites, and alpha-linolenic acid metabolism, were up-regulated in YQ compared to DT (Fig. 4D). Consistent with previous study, flavonoids are one of the most important and abundant compounds in daylily, and can be divided into different classes. These classes mainly include flavanones, flavones, isoflavones, flavanols, dihydroflavonols, anthocyanidins, proanthocyanidins, and auronones based on the saturation, substitution and degree of oxidation of the C-ring (Ma et al., 2023). As shown in Fig. 5A, for flavone and flavonol biosynthesis, the levels of cosmosiin, kaempferin, isovitexin, vitexin, trifolin in DT

were significantly lower than those in YQ. In addition, there were 9 metabolites (2-hydroxycinnamic acid\*, 1-O-galloyl- $\beta$ -D-glucose, 3,4-dihydrocoumarin, gallic acid, cinnamic acid, nicotianamine, Coniferyl alcohol, *cis*-coumarinic acid- $\beta$ -D-glucoside and phosphoenolpyruvate) that participated in the biosynthesis of various plant secondary metabolites and 5 metabolites (9-hydroperoxy-10E,12,15Z-octadecatrienoic acid, 13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid, 12-Oxo-phytyldienoic acid, Jasmonic acid and 9-hydroxy-10, 12, 15-octadecatrienoic acid) involved in alpha-linolenic acid metabolism with higher levels in YQ than those in DT (Fig. 5B-C).

The Pearson correlation coefficient method was adopted to calculate the relationship between differential metabolites and environmental factors (Fig. S4A). The results indicated that the levels of the above metabolites were positively related to daily average and maximum temperatures, available selenium and organic matter, and negatively related to altitude and available potassium. The analysis of environmental factors in the two regions revealed that the altitude and available potassium were substantially lower in YQ than in DT. In contrast, the daily average and maximum temperatures, available selenium, and organic matter were significantly higher in YQ (Table S1-2). Therefore,

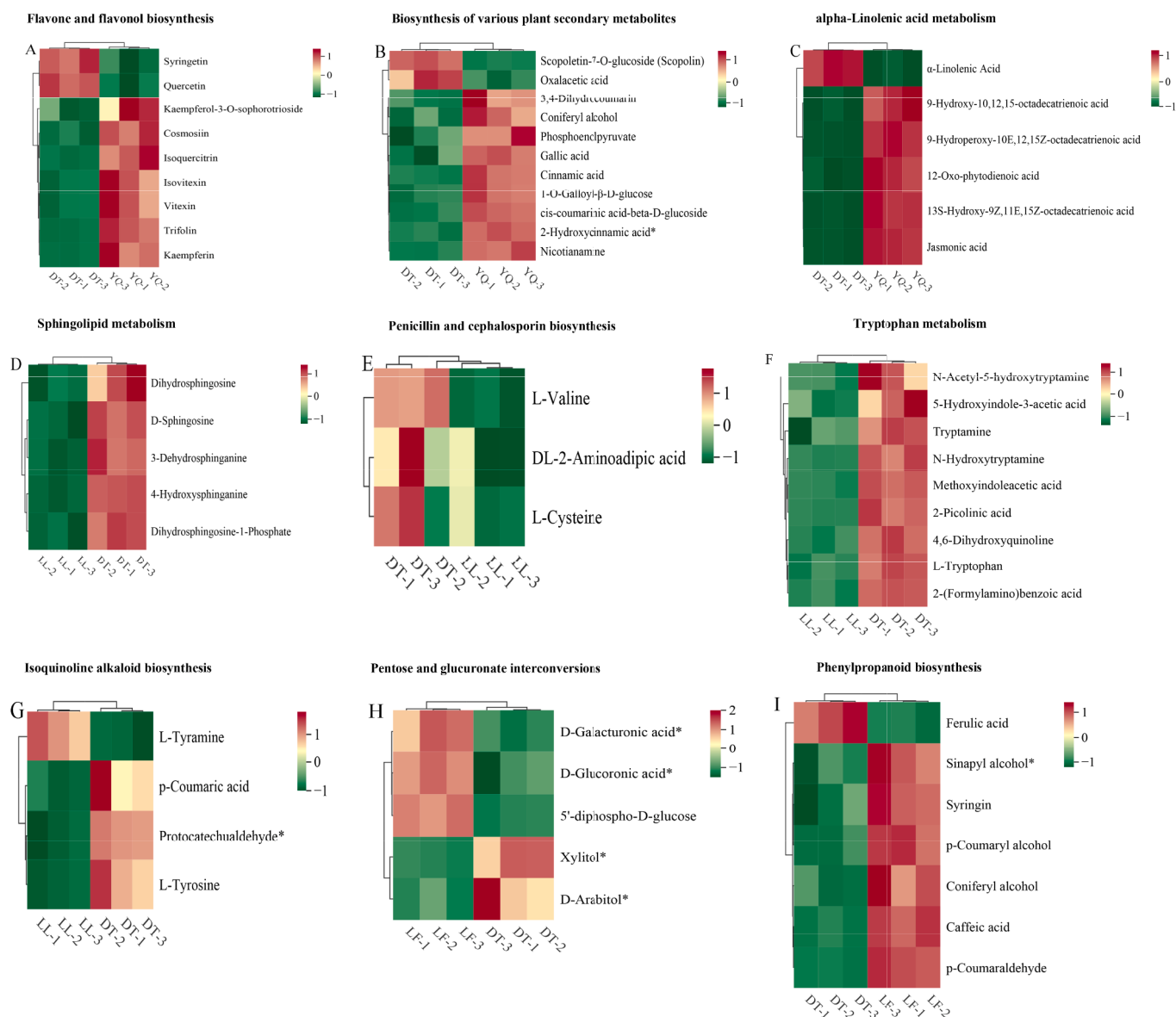


Fig. 5. The heatmap of metabolites level involved in key differential metabolic pathways of DT vs. YQ (A-C), DT vs. LL (D-G), and DT vs. LF (H-I).



the environment in YQ was more conducive to enriching the above-mentioned differential metabolites in daylily.

In particular, vitexin and isovitexin are active components of many traditional Chinese medicines and found in various medicinal plants. In recent years, vitexin has attracted increasing attention for its broad range of pharmacological effects, including antioxidant, anticancer, anti-inflammatory, anti-hyperalgesic, and neuroprotective properties (He et al., 2016). Isoquercitrin could be an effective therapeutic candidate against streptozotocin-induced neurotoxicity and Alzheimer's disease-like changes (Bondonno et al., 2020; Chen et al., 2020).

In addition, the flavonoids and isoflavones in daylily could contribute to its flavor profiles, such as bitter, sour, or astringent (Fenwick et al., 1983). Therefore, the enrichment of flavonoids helps daylily to exert physiological functions and enhance flavor. Also, flavonoids are responsible for the antidepressant, sleep-improving, and lactation-promoting effects of daylily (Guo et al., 2023; Liang et al., 2023). Although the TFC in YQ was lower than that in DT, the content of flavonoid metabolites enriched in the KEGG metabolic pathway was higher than that in DT. Moreover, plant secondary metabolites significantly promote plants' survival and establish ecological links with other species. Secondary metabolites help to protect plants against pathogenic attack and environmental stress (Jan, Asaf, Numan, & Lubna, 2021). In plants, alpha-linolenic acid regulates immunological functions and is involved in gene interaction for growth (Leung et al., 2022). Therefore, the enrichment of the metabolites mentioned above in the daylily of YQ may lead to its good physiological functions and characteristics of coping with environmental stress.

### 3.6.2. Metabolite differences between DT vs. LL

A total of 667 metabolites were identified in DT vs. LL, including 39 alkaloids, 242 amino acids and derivatives, 4 benzene and substituted derivatives, 89 flavonoids, 20 lignans and coumarins, 88 lipids, 24 nucleotides and derivatives, 26 organic acids, 35 others, 77 phenolic acids, 14 quinones, 8 terpenoids, 1 tannin (Table S7). The differential metabolites in the DT and LL regions were mainly concentrated in the sphingolipid metabolism, isoquinoline alkaloid biosynthesis, tryptophan metabolism, and penicillin and cephalosporin biosynthesis pathways, including 5, 4, 9, and 3 metabolites, with the contents of 12 metabolites increased and 9 metabolites decreased (Fig. 4B, Table S9). The DA score plot indicated that all four pathways were downregulated in LL compared to DT (Fig. 4E). In our study, the levels of metabolites that participated in the sphingolipid pathway (such as sphingosine and its derivatives and sphingosine 1-phosphate derivatives) and metabolites involved in penicillin and cephalosporin biosynthesis (such as cysteine and valine) were significantly higher in DT than in LL (Fig. 5D-E). In addition, the levels of tryptophan pathway metabolites and 3 out of 4 metabolites in isoquinoline alkaloid biosynthesis were higher in DT than in LL (Fig. 5F-G).

The correlation between differential metabolites and environmental factors was analyzed (Fig. S4B). The results showed that except for DL-2-Aminoapic acid, L-cysteine, and L-tyramine, the levels of differential metabolites in daylily between LL and DT regions were negatively correlated with altitude, daily average and maximum temperatures, available potassium, available selenium, and organic matter. The soil nutrients (available potassium, available selenium and organic matter) and climate factors (altitude, daily average and maximum temperatures) in LL were higher than in DT (Table S1-2). Therefore, compared to DT, environmental factors led to less accumulation of the metabolites as mentioned above in daylily of the LL area.

Sphingolipids are involved in regulating plant development, perception of stimulus, and response to stress (Liu, Hou, Bao, Wang, & Chen, 2021). In addition, sphingolipids are essential metabolites found in all plant species. They are required to maintain plasma membrane integrity, tolerate and respond to biotic and abiotic stresses, and for intracellular signals (Haslam & Feussner, 2022). Thus, sphingolipid metabolism is essential for plant growth metabolism. In plants,

sphingosine-1-phosphate, a type of sphingosine derivative, has been shown to influence stomatal opening via the abscisic acid signal pathway (Coursol et al., 2003). Free sphingolipids, which include sphingosine, are always signaling molecules and serve as second messengers that regulate stress reactions (Liu et al., 2021). In addition, cysteine is helpful in synthesizing glutathione from glycine and glutamate and is a basic amino acid for protein synthesis. Glutathione is a key antioxidant in plants (Liu & Lin, 2020). For the mammary glands and ovaries, valine is a critical growth factor. It is essential for preventing disorders of the nervous and digestive systems (De & De, 2019). L-tryptophan is an essential amino acid required for protein biosynthesis and has a significant impact on mammal physiology, including gastrointestinal function, immunity, metabolism, and the nervous system (Modoux, Rolhion, Mani, & Sokol, 2021). For example, sleep plays a regulatory role in the maintenance of metabolic homeostasis and the functioning of cells. Tryptophan metabolism is found to be altered in sleep disorders (Bhat, Pires, Tan, Babu, & Guillemain, 2020). In addition, tryptamine in plants is the precursor of the metabolic pathway that induces the production of indole alkaloids, for example, vincristine and vinblastine. These indole alkaloids are important for human health. Moreover, tryptamine derivatives such as serotonin (5-hydroxytryptamine) may participate in many processes, including protecting reactive oxygen species and biological and abiotic stress responses (Negri, Commisso, Avesani, & Guzzo, 2021). Isoquinoline alkaloids have various medicinal properties, including antiviral, antifungal, anti-carcinogenic, anti-oxidative, anti-convulsant, and enzyme inhibiting (Dey et al., 2020). Compared to LL, daylily in DT had a stronger effect in promoting sleep, combating environmental stress and stress, and exerting physiological effects.

### 3.6.3. Metabolite differences between DT vs. LF

As shown in Table S8, 359 metabolites were identified in DT vs. LF, including 20 alkaloids, 105 amino acids and derivatives, 2 benzene and substituted derivatives, 60 flavonoids, 13 lignans and coumarins, 29 lipids, 10 nucleotides and derivatives, 18 organic acids, 25 others, 62 phenolic acids, 11 quinones, 4 terpenoids. We then performed the KEGG enrichment analysis, and the results showed that the phenylpropanoid biosynthesis and pentose and glucuronate conversion pathway were two pathways responding to metabolite difference between the DT and LF regions, including 7 and 5 metabolites, respectively (Fig. 4C, Table S9), with a total of 9 metabolites upregulated and 3 metabolites downregulated. The DA score plot showed that both pathways were upregulated in LF compared to DT (Fig. 4F). In our study, the levels of 3 out of 5 metabolites in pentose and glucuronate interconversions and 6 out of 7 metabolites in phenylpropanoid biosynthesis (i.e., caffeic acid, syringin) were higher than those in DT (Fig. 5H-I).

As shown in Fig. S4C, except for ferulic acid, xylitol \*, and D-arabitol \*, the differential metabolites between the two regions were positively correlated with daily average and maximum temperatures and negatively correlated with altitude, available potassium and organic matter. The altitude and soil nutrients (available potassium and organic matter) in LF were significantly lower than those in DT (Table S2). The daily average and maximum temperatures in LF were higher than in DT. Therefore, the environment in LF is more conducive to enriching the above-mentioned differential metabolites in daylily.

Phenylpropanoids are crucial to plant growth and development and in response to environmental stimuli, for example, tolerance and resistance to abiotic/biotic stress (Rahim, Zhang, & Busatto, 2023). In addition, this metabolic pathway plays a vital role in the biosynthesis of numerous compounds, including flavonoids (anthocyanins, flavonols, etc.), lignin, coumarins, and lignans (Fraser & Chapple, 2011). Also, several phenylpropanoid compounds could act as potent antioxidants to exert significant human health benefits. Caffeic acid (CA) is the primary component of hydroxycinnamic acid, which has the functions of antioxidant, anticancer, antidiabetic, antihypertensive, antimicrobial, hepatoprotective, antiviral, etc. They are present in most botanicals (Mohd et al., 2022). Moreover, syringin showed anti-inflammatory and

antioxidant potential in asthma (Dai, Niu, Wang, & Wang, 2021). When plants face environmental stress, the pentose and glucuronate interconversions are enhanced, promoting carbohydrate and energy metabolism and inducing the synthesis of secondary metabolites (Sun et al., 2018). That is to say, compared to the daylily in DT, the daylily in LF had better tolerance to abiotic and biotic stress, and better physiological activity.

#### 4. Conclusion

In this study, we investigated the protein, TPCs, TFCs, and amino acid content of daylily in different producing areas. Protein and amino acid contents were the lowest in DT, while the moisture and TPCs were the highest. Protein and amino acid contents were highest in YQ, which also had the highest umami and sweet amino acid content, while TFC was highest in LL. Then, we used the RDA analysis to identify and discover the key environmental factors affecting the nutritional differences of daylily in different regions: altitude and temperature. A UPLC-MS/MS system was conducted to qualitatively and quantitatively reveal the metabolite differences of daylily from different producing areas. A total of 1672 metabolites were detected in daylily, among which the content of amino acids and derivatives was the highest, followed by flavonoids, phenolic acids, and lipids. Significant differences in metabolite accumulation between DT and YQ, LL, LF were observed by the PCA and OPLS-DA score plots. The differential metabolites between DT and YQ, LL, and LF were 557, 667, and 359, respectively. Also, the differential metabolic pathways and metabolites between DT and YQ, LL and LF were analyzed. The differences in KEGG metabolic pathways between DT and LL (sphingolipid metabolism, isoquinoline alkaloid biosynthesis, tryptophan metabolism, and penicillin and cephalosporin biosynthesis pathways), were downregulated. In contrast, the differential metabolic pathways between DT and YQ (flavone and flavonol biosynthesis, biosynthesis of variable plant secondary metabolites, and alpha-linolenic acid metabolism), DT and LF (phenolpropanoid biosynthesis and pentose and glucose conversion pathways), were up-regulated, suggesting that daylily in the YQ and LF regions may have better tolerance to abiotic and biotic stresses, and physiological activity. Therefore, the YQ and LF regions are also suitable for cultivating daylily with high nutritional value.

Our study comprehensively determined the nutritional components and metabolites of daylily and analyzed the differences in nutrient composition and functional constituents of daylily in different regions of Shanxi province from the perspective of critical environmental factors. The results provide an important basis for developing daylily as a functional food.

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#### CRediT authorship contribution statement

**Haizhen Wang:** Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Mengying Zhao:** Conceptualization, Investigation. **Zhen Zhen Wu:** Conceptualization, Investigation. **Nannan Qin:** Conceptualization, Investigation. **Yongxia Fu:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Shang Guo:** Conceptualization, Funding acquisition, Project administration, Supervision, 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.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Fig.S1: RDA plot showing the relationships between environmental factors and nutrition composition in daylily of four regions of Shanxi Province. Fig.S2: Venn diagram shows the overlapping and unique metabolites amongst the comparison groups. Fig.S3: Nutrient content distribution of daylily obtained from four producing areas in Shanxi Province. Fig.S4: Correlation analysis between differential metabolites and soil nutrients and climate factors. Table S1: The climate factors of different daylily-producing areas in Shanxi Province. Table S2: The soil nutrients of different daylily-producing areas in Shanxi province. Table S3: The proximate composition of daylily in different producing areas of Shanxi province. The following supplementary tables are available at the database Dryad, with a link of DOI: 10.5061/dryad.dbrv15f77. Table S4: Information of metabolites identified in daylily. Table S5: Information of metabolites identified in daylily of all three comparison groups. Table S6: Information of metabolites identified in daylily from DT and YQ. Table S7: Information of metabolites identified in daylily from DT and LL. Table S8: Information of metabolites identified in daylily from DT and LF. Table S9: The KEGG pathways responding to the differential metabolites among all three comparison groups. Supplementary data to this article can be found online at <http://doi.org/10.1016/j.fochx.2024.101239>.

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