Metabolomic Analysis of Flower Color Compounds in Three Rose Cultivars: Postprint

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Abstract

Rose (Rosa rugosa) possesses high ornamental and commercial value, but its relatively monotonous flower color limits its development, utilization, and application in landscape design. To investigate the color-producing substances in three different rose varieties—'Kushui Rose', 'Mohong Rose', and 'Bulgarian White Rose'—this study utilized Ultra-Performance Liquid Chromatography-Quadrupole-Time-of-Flight Mass Spectrometry (UPLC-Q-TOF-MS) to detect the types and contents of flavonoids in petals, conducted enrichment analysis of differential metabolites through the KEGG database to screen key metabolites, and analyzed their correlation with flower color phenotypic values. The results showed that: (1) A total of 58 metabolites were detected in the petals of the three different colored rose varieties, among which there was only one anthocyanin, cyanidin-3-O-glucoside, accounting for approximately 30.45%; (2) K-means cluster analysis indicated that 12 key metabolites were annotated to KEGG metabolic pathways, among which pinocembrin and myricetin were the main substances determining the red coloration of 'Kushui Rose' and 'Mohong Rose', while eriodictyol, luteolin, and kaempferol were the main substances determining the white coloration of 'Bulgarian White Rose'. These findings can provide a theoretical basis for breeding roses with specific colors and promote the application of roses in landscaping.

Full Text

Metabolomics Analysis of Flower Color Substances in Three *Rosa rugosa* Cultivars

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Abstract

Rosa rugosa is a deciduous shrub in the Rosaceae family with high ornamental and commercial value, but its limited color range restricts its development and landscape applications. This study investigated the color-forming substances in three distinct rose cultivars: 'Kushui Rose' (*Rosa rugosa* × *Rosa sertata*), 'Crimson Glory' (*Rosa* 'Crimson Glory'), and 'Bulgarian White Rose' (*Rosa alba*). Using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), we identified and quantified flavonoid compounds in petal samples. Differential metabolites were enriched and analyzed via the KEGG database to screen key metabolites and examine their correlation with flower color phenotypes. The results revealed: (1) A total of 58 metabolites were detected across the three cultivars, with only one anthocyanin—cyanidin-3-O-glucoside—accounting for approximately 30.45% of total flavonoids; (2) K-means clustering analysis identified 12 key metabolites mapped to KEGG pathways, with pinocembrin and myricetin as the primary determinants of red coloration in 'Kushui Rose' and 'Crimson Glory', while eriodictyol, luteolin, and kaempferol were the main compounds responsible for the white coloration of 'Bulgarian White Rose'. These findings provide a theoretical foundation for breeding rose cultivars with specific colors and promote their application in landscape horticulture.

Keywords: *Rosa rugosa* cultivars, flower color substances, metabolomics, UPLC-Q-TOF-MS, correlation

Introduction

Flower color represents a critical trait in ornamental plants, determining both aesthetic and commercial value [**?**]. Recent research has demonstrated that plant pigment types and contents are the primary factors influencing flower color formation. The main pigment categories include flavonoids, carotenoids, and alkaloids [**?**, **?**], with flavonoid secondary metabolites playing a particularly important role in color development. Anthocyanins, which occur as glycosylated anthocyanidins, constitute the primary pigments for red, blue, and purple colors in petals, while chalcones contribute to yellow coloration. In contrast, flavones and flavonols are typically colorless or pale white [**?**, **?**]. Studies in Ericaceae species have shown that flower color depends on flavonoid composition, with anthocyanins exerting decisive effects and flavonols serving auxiliary color functions [**?**, **?**]. Advances in plant metabolomics have elucidated coloration mechanisms in numerous ornamentals, including camellia (*Camellia japonica*), mountain cherry (*Prunus serrulata*), and cherry (*P. pseudocerasus*) [**?**], as well as sunflower (*Helianthus annuus*) [**?**]. Shi et al. [**?**] employed metabolomics to clarify how different metabolites and pathways regulate color differences between Yunnan red and Crimson Glory roses. Collectively, these studies indicate that pigment types, contents, and biosynthetic pathways are crucial determi-

nants of floral color diversity [**?**, **?**].

Rosa rugosa, a deciduous shrub native to China with a long cultivation history, is renowned as the "Queen of Flowers" and "Flower of Love." This multifunctional plant serves ornamental, economic, ecological, and culinary purposes and is listed as a second-class protected species in China [**?**]. Chinese rose germplasm is remarkably diverse, encompassing single, semi-double, and double-flowered forms, including the 'Kushui Rose' (*Rosa rugosa* × *Rosa sertata*), a natural hybrid between traditional Chinese rose and *Rosa sertata* characterized by numerous small magenta flowers with fragrance and high oil yield [**?**]; 'Crimson Glory' (*Rosa* 'Crimson Glory'), a hybrid between Hybrid Tea and Hybrid Perpetual roses with deep red flowers, extended flowering period, and strong fragrance, widely cultivated as the main edible rose variety in Yunnan [**?**]; and 'Bulgarian White Rose' (*Rosa alba*), also known as the Damask rose, producing pink to white flowers with high yield and oil quality [**?**]. However, as an important ornamental species, roses predominantly exhibit red, pink, white, and purple colors, with rare variants, severely limiting their landscape applications [**?**]. Previous research has focused on essential oil extraction, resource conservation, genetic diversity analysis, food production, transgenic color regulation, and propagation techniques [**?**]. Although transgenic approaches for color modification have been reported, studies on the biochemical basis of edible rose coloration and pigment composition remain incomplete, hindering the development and utilization of rose pigments in horticulture [**?**, **?**].

This study targeted three edible double-flowered rose cultivars with distinct colors—'Kushui Rose', 'Crimson Glory', and 'Bulgarian White Rose'—to investigate key metabolites influencing coloration through targeted metabolomics. Specifically, we aimed to: (1) analyze differences in flavonoid types and contents among the three cultivars; (2) screen differential metabolites and pathways to identify primary color-determining compounds. These findings will provide theoretical support for breeding novel rose colors and expand their landscape applications.

Materials and Methods

1.1 Material Collection and Processing Experimental materials comprised three cultivated rose varieties: magenta 'Kushui Rose' (*Rosa rugosa* × *Rosa sertata*, KSMG), red 'Crimson Glory' (*Rosa* 'Crimson Glory', MHMG), and white 'Bulgarian White Rose' (*Rosa alba*, BMG). Petal samples were collected from the Rose Germplasm Resource Base at the Yongdeng County Rose Research Institute in Gansu Province. For each variety, three healthy, uniformly managed plants were selected. Fully opened flowers were sampled on May 20 and June 5, 2022 [Figure 1: see original paper]. During collection, petals were sampled from four directional orientations per plant, then pooled. Each variety was analyzed with three biological replicates. Samples were

immediately placed in numbered sealed bags, stored in ice boxes to prevent wilting, and subsequently frozen at -80°C for metabolomic analysis [**?**].

1.2.1 Flower Color Phenotype Measurement Color parameters were measured using the CIE L*ab** color space system [**?**, **?**]. Fresh petals were analyzed with a WR18 precision colorimeter (Shenzhen Weifu Optoelectronics Technology Co., Ltd.) to determine lightness (L*), redness (a*), and yellowness (b*) values, from which chroma (C)* and hue angle (h°) were calculated. Under C/2° illumination, the measuring aperture was positioned at the central upper epidermis of each petal. Three flowers per sample were measured, with values averaged [**?**].

1.2.2 Flavonoid Extraction Fresh petals were freeze-dried at -80°C and pulverized (60 Hz, 30 s). A 100 mg sample was transferred to a 5 mL centrifuge tube with 3,000 L extraction solvent (75% methanol containing 1% acetic acid), vortexed for 30 s, homogenized at 40 Hz for 4 min, and sonicated in an ice-water bath for 30 min. After centrifugation at 12,000 rpm (13,800 × g, 8.6 cm radius) for 15 min at 4°C, 2,500 L supernatant was collected, dried under nitrogen, and reconstituted in 1,500 L solvent B (50% methanol with 0.1% formic acid containing internal standard). Following 1 min vortexing and 15 min ice-water bath sonication, samples were centrifuged again at 12,000 rpm for 15 min at 4°C. The supernatant was filtered through a 0.22 m membrane into 2 mL vials. Quality control (QC) samples were prepared by mixing equal aliquots from all samples prior to instrumental analysis [**?**, **?**, **?**].

1.2.3 Flavonoid Qualitative and Quantitative Analysis Flavonoids were analyzed using UPLC-Q-TOF-MS technology. The system comprised an ACQUITY™ UPLC I-Class chromatograph (Waters Corporation, Milford, MA, USA) coupled with an Xevo G2-XS QTof MS mass spectrometer (Waters Corporation, Manchester, UK) controlled by UNIFI 1.8 software. Chromatographic separation employed a Waters UPLC BEH C18 column (1.7 m, 2.1 mm × 150 mm) with mobile phases of 0.1% formic acid in water (A) and acetonitrile (B) at 0.3 mL · min^{-1}. The gradient elution program was: 0–0.5 min, 10% B; 0.5–15 min, 10–60% B; 15–16.01 min, 60–98% B; 16.01–18.00 min, 98% B; 18.00–18.01 min, 98–10% B; 18.01–20 min, 10% B. Column temperature was 40°C, autosampler temperature 8°C, and injection volume 2 L. Mass spectrometry data were acquired in multiple reaction monitoring (MRM) mode [**?**].

1.3 Data Analysis Color parameters (L*, a*, b**) were used to calculate chroma ($C^* = \sqrt{a^{*2} + b^{*2}}$) and hue angle ($h^\circ = \arctan(a^*/b^*)$). Correlations between color indices and key metabolites were analyzed using SPSS 22.0. Flavonoid metabolites were quantified via the UPLC-MS/MS platform of Shanghai Biotree Biomedical Technology Co., Ltd. MetaboAnalyst 5.0 software performed multivariate statistical analysis of metabolites across samples in unsupervised mode,

with differential metabolites selected based on $P < 0.05$ and VIP ≥ 1. Pathway enrichment analysis utilized the KEGG database, MBROLE 2.0, and the Microbiome Analysis website.

Results

2.1 Flower Color Characteristics of Three Rose Cultivars In the CIE L*ab** color space, L* represents lightness, with higher values indicating brighter petals. As shown in , 'Bulgarian White Rose' exhibited the highest L* value (78.41), indicating the brightest coloration. The a* parameter ranges from positive (red) to negative (green) values; 'Crimson Glory' showed an a* value 106.46 units higher than 'Bulgarian White Rose', confirming its red hue. The b* parameter transitions from positive (yellow) to negative (blue); 'Bulgarian White Rose' displayed intermediate b* values, appearing white with yellowish undertones. Chroma (C*) indicates color saturation, with 'Crimson Glory' showing the maximum C* value (110.37), reflecting deep red coloration, followed by 'Kushui Rose' (C* = 71.60) with magenta tones. Hue angle (h°) describes color progression through red (0°), orange, yellow (~90°), green, cyan, blue, and purple (270–360°). 'Crimson Glory' and 'Bulgarian White Rose' exhibited h° values between 0–90°, placing them in the red-yellow range, whereas 'Kushui Rose' fell within 270–360°, passing through the purple region.

2.2 Flavonoid Composition and Content in Rose Petals A total of 58 flavonoid metabolites were detected across the three cultivars, comprising 24 flavones (~18.85%), 9 flavonols (~31.89%), 1 anthocyanin (~30.45%), 8 flavanols (~14.49%), 6 dihydroflavones (~0.08%), 3 isoflavones, 2 chalcones (~0.09%), and 5 other polyphenolic compounds (~4.15%). Hierarchical clustering analysis revealed distinct flavonoid accumulation patterns among the three cultivars [Figure 2: see original paper]. After normalization, color intensity in the heatmap indicated metabolite abundance, with red representing high content and blue representing low content.

2.3 Differential Metabolite Screening Analysis Differential flavonoid metabolites were screened using criteria of $P < 0.05$ and VIP ≥ 1. As illustrated in [Figure 3: see original paper]A–C, 45 differential metabolites were identified between 'Crimson Glory' and 'Bulgarian White Rose', with 22 significantly upregulated and 10 downregulated. Between 'Bulgarian White Rose' and 'Kushui Rose', 41 differential metabolites were found (8 upregulated, 22 downregulated). The comparison between 'Crimson Glory' and 'Kushui Rose' also revealed 41 differential metabolites (11 upregulated, 14 downregulated).

To examine flavonoid variation trends across color gradients, z-score normalization was applied to mean relative abundances of differential metabolites, followed by K-means clustering. During the transition from white to magenta

to deep red , 33 metabolites showed increasing trends while 8 exhibited decreasing trends. Notably, cyanidin-3-O-glucoside (the sole anthocyanin) displayed a consistent upward trend, suggesting its primary role in red coloration. Flavones and flavonols showed variable trends, with some increasing and others decreasing across color groups.

2.4 KEGG Functional Annotation and Enrichment Analysis of Differential Metabolites Enrichment analysis of the increasing and decreasing flavonoid metabolites from K-means clustering identified 12 key metabolites successfully annotated to KEGG pathways. These key differential metabolites participated in multiple metabolic pathways, with some involved in two or more pathways . Among the 33 increasing metabolites, 9 were annotated, including: vitexin and pinocembrin in flavonoid biosynthesis; eriodictyol and (-)-epigallocatechin in flavonoid biosynthesis and secondary metabolite biosynthesis; myricetin in flavonoid biosynthesis, flavone/flavonol biosynthesis, and secondary metabolite biosynthesis; naringenin, cianidanol, and chalconaringenin in flavonoid biosynthesis, secondary metabolite biosynthesis, phenylpropanoid biosynthesis, and metabolic pathways; and apigenin in five pathways including flavonoid biosynthesis, flavone/flavonol biosynthesis, secondary metabolite biosynthesis, phenylpropanoid biosynthesis, and metabolic pathways. Among the 8 decreasing metabolites, only 3 were annotated: quercetin, kaempferol, and luteolin, all participating in flavonoid biosynthesis, flavone/flavonol biosynthesis, secondary metabolite biosynthesis, and metabolic pathways.

2.6 Relationship Between Petal Phenotype and Key Metabolite Content Correlation analysis was performed between color parameters and the 12 key metabolites successfully annotated to flavonoid pathways (9 increasing and 3 decreasing trends). As shown in , lightness (L*) was extremely significantly negatively correlated with redness (a*) and chroma (C*) ($P < 0.01$), indicating that increased brightness shifts petal color toward white. Pinocembrin and myricetin were significantly negatively correlated with L* ($P < 0.01$ and $P < 0.05$, respectively) and positively correlated with a* and C* ($P < 0.01$), demonstrating that their accumulation enhances color intensity and reduces brightness, promoting red coloration. Conversely, eriodictyol, luteolin, and kaempferol were significantly positively correlated with L* and negatively correlated with a* and C* ($P < 0.01$), indicating their accumulation increases brightness and whiteness. Luteolin showed positive correlation with b* and negative correlation with h°, suggesting its accumulation shifts color toward yellow. Hue angle was significantly negatively correlated with a* and C* ($P < 0.01$), confirming that increased redness reduces hue angle, moving color toward the red region. These results identify pinocembrin and myricetin as primary determinants of red coloration, while eriodictyol, luteolin, and kaempferol are key compounds for white coloration, with luteolin specifically contributing to yellow undertones in 'Bulgarian White Rose'.

Discussion and Conclusion

Flavonoids constitute primary pigments in flower coloration, with anthocyanins playing a pivotal role. Anthocyanin content directly influences flower color [**?**]. Cyanidin and its derivatives are widely reported in red petals [**?**]. Li et al. [**?**] identified cyanidin-3-O-glucoside as the main anthocyanin in red camellia, and Du et al. [**?**] found cyanidin to be most abundant in red-flowered rhododendrons among 30 color variants. These findings align with our results. Previous studies identified cyanidin as the predominant flavonoid in Crimson Glory rose, with significantly higher content than other compounds [**?**], and as the main pigment component in Kushui rose [**?**, **?**]. Our detection of 58 flavonoid metabolites across the three cultivars, with cyanidin-3-O-glucoside as the sole anthocyanin (~30.45% of total flavonoids), confirms its central role. Cyanidin-3-O-glucoside accounted for 47.75% and 15.55% of total flavonoids in Crimson Glory and Kushui roses, respectively, compared to only 0.04% in Bulgarian White Rose, establishing it as the primary red pigment.

Further investigation of color-determining metabolites revealed that pigment composition critically affects flower color [**?**, **?**]. White-flowered 'Zizhi' rose contains only flavones, whereas pink and purple variants contain both flavones and anthocyanins [**?**]. In white camellia cultivars, luteolin and quercetin-3-O-glucoside are the most abundant flavonoids [**?**], and white roses and chrysanthemums contain only pale yellow or colorless flavones and flavonols [**?**]. Our screening ($P < 0.05$, VIP ≥ 1) identified 12 key metabolites: 6 flavones, 2 flavanols, 2 flavonols, 1 dihydroflavone, and 1 chalcone. Pinocembrin and myricetin accumulation significantly affected L*, a*, and C* values, enhancing color intensity and red hue. In contrast, luteolin, eriodictyol, and kaempferol accumulation increased lightness and whiteness, with luteolin positively correlating with b* and contributing to yellow undertones in Bulgarian White Rose. Thus, pinocembrin, myricetin, luteolin, eriodictyol, and kaempferol are key metabolites determining rose coloration.

In conclusion, significant differences in flavonoid metabolite profiles among 'Kushui Rose', 'Crimson Glory', and 'Bulgarian White Rose' substantially influence flower color. Our results demonstrate that among 58 detected flavonoids, cyanidin-3-O-glucoside, pinocembrin, and myricetin are the primary pigments for red coloration in Kushui and Crimson Glory roses, while eriodictyol, luteolin, and kaempferol are the key compounds for white coloration in Bulgarian White Rose.

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Note: Figure translations are in progress. See original paper for figures.