Metabolomic Analysis of Floral Pigments in Three Rose Varieties: Postprint

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Abstract

Rose (Rosa rugosa) possesses high ornamental and commercial value, but its relatively monotonous flower color restricts its development, utilization, and application in landscape design. To investigate the color-producing compounds in three rose varieties—'Kushui Rose', 'Mohong Rose', and 'Bulgarian White Rose'—this study utilized ultra-high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS) to detect the types and contents of flavonoids in petals, performed 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: (1) A total of 58 metabolites were detected in petals of the three differently colored rose varieties, among which only one anthocyanin, cyanidin-3-O-glucoside, accounted for approximately 30.45%; (2) K-means cluster analysis revealed that 12 key metabolites were annotated to KEGG metabolic pathways, with pinocembrin and myricetin being 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 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 Rose Rugosa Cultivars

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Abstract

Rose (*Rosa rugosa*) possesses high ornamental and commercial value, but its relatively limited color range restricts its development, utilization, and application in landscape architecture. To investigate the color-determining substances in three distinct rose varieties—'Kushui Rose', 'Crimson Glory', and 'Bulgarian White Rose'—this study employed ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) to detect the types and contents of flavonoids in petals. Differential metabolites were subjected to enrichment analysis using the KEGG database to screen for key metabolites and analyze their correlation with flower color phenotypic values. The results revealed: (1) A total of 58 metabolites were detected across the three different colored rose petals, with only one anthocyanin—cyanidin-3-O-glucoside—accounting for approximately 30.45% of the total; (2) K-means clustering analysis identified 12 key metabolites annotated to KEGG metabolic pathways, among which pinocembrin and myricetin were the primary substances determining the red coloration of 'Kushui Rose' and 'Crimson Glory', while eriodictyol, luteolin, and kaempferol were the main substances responsible for the white coloration of 'Bulgarian White Rose'. These findings provide a theoretical basis for breeding roses with specific colors and promote their application in landscape greening.

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

Introduction

Flower color represents a crucial characteristic of ornamental plants, determining both their aesthetic and commercial value (Huang, 2017). Recent research has demonstrated that the primary factors influencing flower color formation are the types and contents of plant pigments, which mainly include three major categories: flavonoids, carotenoids, and alkaloids (Li et al., 2021; Zhao et al., 2021). Among these, the accumulation of flavonoid secondary metabolites constitutes a key factor affecting color development. Anthocyanins, which appear as glycosylated anthocyanidins in plants, constitute the main components responsible for red, blue, and purple colors in petals, while chalcones represent important color constituents in yellow flowers. Flavones and flavonols, however, are typically colorless or pale white (Zhou et al., 2009; Lin et al., 2021). Studies on Ericaceae species have revealed that flower color depends on flavonoid types, with anthocyanins playing a critical role and flavonols serving auxiliary color functions (Wang et al., 2020; Zhang et al., 2022). With recent advances in plant metabolomics technology, preliminary investigations into coloration mechanisms have been conducted in numerous ornamental plants, including camellia (*Camellia japonica*), mountain cherry (*Prunus serrulata*), and cherry (*P. pseudocerasus*) (Guo et al., 2018), as well as sunflower (*Helianthus annuus*) (Zhao et al., 2021). Shi et al. (2020) utilized metabolomics to elucidate how different metabolites and metabolic pathways regulate color differences between Yunnan red and Crimson Glory roses. Collectively, current research indicates that pigment types, contents, and metabolic synthesis pathways represent important factors influencing floral color diversity (Fu et al., 2021; Wei, 2023).

Rose (*Rosa rugosa*), a deciduous shrub belonging to the genus *Rosa* in the Rosaceae family, is native to China with a long cultivation history. Revered as the "Queen of Flowers" and the "Flower of Love," this plant integrates ornamental, economic, ecological, and edible functions, and has been designated as a second-class protected species in China (Zhang, 2015). Chinese rose cultivars are extremely diverse, encompassing double-petaled, single-petaled, and semi-double varieties, as well as cut flower types. Notable cultivars include Gansu's 'Kushui Rose' (*Rosa rugosa* × *Rosa sertata*), a natural hybrid between traditional Chinese rose and *R. sertata*, characterized by numerous small magenta flowers with fragrance and high oil yield, serving as an important edible double-petaled variety (Wu, 2020); 'Crimson Glory' (*Rosa Crimson Glory*), also known as 'Zhumo Shuanghui', a hybrid between Hybrid Tea Rose and Hybrid Perpetual Rose, featuring deep red flowers with extended blooming period, intense fragrance, and high yield, widely cultivated as a primary edible rose variety in Yunnan (Ning et al., 2021); and 'Bulgarian White Rose' (*Rosa alba*), also called 'Turkish Rose', producing pale pink, pink, and white flowers with high yield, high oil content, and excellent quality, representing another premium edible rose variety for essential oil extraction and rose water processing (Zhao, 2017). However, as an important ornamental plant, roses predominantly exhibit red, pink, white, and purple colors, with other colors rarely observed, significantly limiting their application in landscape greening (Cheng et al., 2021).

Current domestic and international research on roses has primarily focused on volatile oil extraction and utilization, rose resource development and conservation, genetic diversity analysis and molecular markers, rose-related food production, transgenic color regulation technology, and cultivation and propagation techniques (Zou, 2018). Although several studies have reported on transgenic color regulation in roses with certain achievements, research on edible rose color formation and pigment composition remains incomplete, and systematic documentation of the material basis for rose coloration is lacking. These gaps hinder the development and utilization of rose pigments and their application in landscape greening (Xu et al., 2011; Yang et al., 2011).

This study investigated three edible double-petaled rose cultivars with different flower colors—'Kushui Rose', 'Crimson Glory', and 'Bulgarian White Rose'—using targeted metabolomics technology to explore key metabolite components affecting coloration. The research addressed two primary questions: (1) analyzing differences in flavonoid compound types and contents among the three rose varieties; and (2) screening differential metabolites and metabolic pathways across cultivars to identify the main metabolite components influencing rose coloration. These findings provide a theoretical foundation for breeding roses with specific colors and promote their application in landscape greening.

Materials and Methods

1.1 Material Collection and Processing The 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 materials were collected from the rose germplasm resource base at the Yongdeng County Rose Research Institute in Gansu Province. For each material, three uniformly growing, disease-free plants under consistent management were selected. Fully bloomed petal samples were collected on May 20 and June 5, 2022 [Figure 1: see original paper]. During collection, different petal samples were taken from four directional orientations and then mixed, with three biological replicates performed for each variety. After sampling, petal specimens were placed in numbered sealed bags, stored in ice boxes to prevent wilting, and subsequently preserved at -80 °C for subsequent metabolomics analysis (Wang et al., 2017).

1.2.1 Flower Color Phenotype Measurement Flower color was measured using the CIE L*ab** color system established by the International Commission on Illumination (Wang et al., 2017; Li et al., 2019). Fresh petals were collected, and the brightness (L*), redness (a*), and yellowness (b*) parameters were determined using a WR18 precision colorimeter (Shenzhen Weifu Optoelectronics Technology Co., Ltd.), from which chroma (C*) and hue angle (h°) were calculated to digitize flower color (Zhang, 2015). Under C/2° illumination, the light collection aperture was aligned with the central region of the petal upper epidermis for measurement, with color parameters measured for three flowers per sample and average values calculated (Wang et al., 2004).

1.2.2 Flavonoid Extraction Fresh petals were freeze-dried at -80 °C and pulverized into powder (60 Hz, 30 s). A 100 mg sample was placed in a 5 mL centrifuge tube, and 3,000 L of extraction solution (75% methanol containing 1% acetic acid) was added. The mixture was vortexed for 30 s, homogenized at 40 Hz for 4 min, and ultrasonicated in an ice-water bath for 30 min. Following centrifugation at 12,000 $\mathrm{r \cdot min^{-1}}$ (relative centrifugal force 13,800 ×g, radius 8.6 cm) for 15 min at 4 °C, 2,500 L of supernatant was collected and dried under nitrogen gas. The residue was reconstituted in 1,500 L of extraction solution B (50% methanol containing 0.1% formic acid with internal standard), vortexed for 1 min, ultrasonicated in an ice-water bath for 15 min, and centrifuged again at 12,000 $\mathrm{r \cdot min^{-1}}$ (13,800 ×g, radius 8.6 cm) for 15 min at 4 °C. The supernatant was filtered through a 0.22 m membrane and transferred to a 2 mL sample vial. Quality control (QC) samples were prepared by uniformly mixing aliquots from all samples for instrumental detection and analysis (Wang et al., 2017; Daneshpajooh et al., 2019; Zhang et al., 2022).

1.2.3 Qualitative and Quantitative Analysis of Flavonoids UPLC-Q-TOF-MS technology was employed for qualitative and quantitative analysis of petal flavonoids. The system consisted of an ACQUITYTM UPLC I-Class ultra-high performance liquid chromatography system (Waters Corporation, Milford, MA, USA), an Xevo G2-XS QTof MS mass spectrometry system (Waters Corporation, Manchester, UK), and UNIFI 1.8 software. Chromatographic separation was performed on a Waters UPLC BEH C18 column (1.7 m, 2.1 mm × 150 mm). The mobile phase comprised 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.3 $\mathrm{mL \cdot min^{-1}}$. 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. The column oven temperature was set at 40 °C, the autosampler temperature at 8 °C, and the injection volume at 2 L. Mass spectrometry data acquisition was conducted in multiple reaction monitoring (MRM) mode (Zhao et al., 2021).

1.3 Data Analysis The CIE L*ab** color system was used to measure rose petal brightness (L*), redness (a*), and yellowness (b*) values, from which chroma* ($C = \sqrt{(a^2 + b^2)}$) and hue angle ($h° = \arctan(a/b)$) were calculated. SPSS 22.0 software was employed for correlation analysis between flower color and key metabolites. Flavonoid metabolites in different colored roses were determined using the UPLC-MS/MS detection platform of Shanghai Biotree Biomedical Technology Co., Ltd. MetaboAnalyst 5.0 software was utilized for qualitative and quantitative analysis of all target compounds. Unsupervised multivariate statistical analysis was performed on metabolites across sample groups, and significantly differential metabolites were selected based on $P < 0.05$ and VIP ≥ 1. Pathway enrichment analysis was conducted using the KEGG database, MBROLE 2.0, and the Microbiome Analysis website.

Results

2.1 Flower Color Characteristics of Three Rose Cultivars In the CIE Lab* color space, L* represents lightness variation, with higher values indicating brighter petals. As shown in Table 1 , 'Bulgarian White Rose' exhibited the highest L* value (78.41), indicating a brighter coloration. The a* parameter ranges from positive (red) to negative (green) values, with 'Crimson Glory' showing an a* value 106.46 units higher than 'Bulgarian White Rose', reflecting its red coloration. The b* parameter transitions from positive (yellow) to negative (blue) values, with 'Bulgarian White Rose' displaying intermediate b* values, resulting in a white color with yellowish undertones. Chroma (C*) indicates color vividness, with higher values representing more saturated colors. 'Crimson Glory' showed the maximum C* value (110.37), corresponding to deep red coloration, followed by 'Kushui Rose' (C* = 71.60) with magenta coloration. The hue angle (h°) describes the seven color regions from red (0°), orange, yellow (90°), green, cyan, blue, to purple (270°–360°). Both 'Crimson Glory' and

'Bulgarian White Rose' exhibited h° values between 0°–90°, placing them in the red-yellow range, whereas 'Kushui Rose' showed h° values between 270°–360°, passing through the purple region.

2.2 Flavonoid Composition and Content in Petals of Three Rose Cultivars As illustrated in Figure 2 [Figure 2: see original paper], a total of 58 flavonoid metabolites were detected across the three rose samples, including 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%). Clustering analysis of petal samples and metabolites revealed significant differences in flavonoid accumulation patterns among the three rose varieties. After normalization, different colors indicate flavonoid metabolite content, with red representing high abundance and blue representing low abundance.

2.3 Screening Analysis of Differential Metabolites Differential flavonoid metabolites among groups were screened based on $P < 0.05$ and $VIP \geq 1$. The fold change represents the ratio of metabolite expression levels between different colored petal samples. As shown in Figure 3 [Figure 3: see original paper]A–C, 45 differential flavonoid metabolites were identified between 'Crimson Glory' and 'Bulgarian White Rose', including 22 significantly upregulated and 10 downregulated metabolites. Between 'Bulgarian White Rose' and 'Kushui Rose', 41 differential metabolites were detected, with 8 upregulated and 22 downregulated. Between 'Crimson Glory' and 'Kushui Rose', 41 differential metabolites were found, comprising 11 upregulated and 14 downregulated compounds.

To investigate variation trends of flavonoid metabolites across different colored roses, the average relative content of all differential metabolites was z-score normalized and subjected to K-means clustering analysis. During the color transition from white to magenta to deep red (Table 2), 33 metabolites showed increasing trends while 8 exhibited decreasing trends. As petal color progressed from white through magenta to deep red, flavone and flavonol contents displayed both increasing and decreasing patterns, whereas cyanidin-3-O-glucoside (the anthocyanin) consistently increased, suggesting it as the primary pigment responsible for red coloration in rose petals.

2.4 KEGG Functional Annotation and Enrichment Analysis of Differential Metabolites Enrichment analysis was performed on flavonoid metabolites showing increasing and decreasing trends from K-means clustering. A total of 12 key metabolites were successfully annotated to KEGG metabolic pathways, with key differential metabolites participating in not just one but potentially multiple metabolic pathways (Table 3). Among the 33 metabolites with increasing trends across the three materials, 9 were annotated, including vitexin and pinocembrin involved in flavonoid biosynthesis; eriodictyol and (-)-epigallocatechin participating in both flavonoid biosynthesis and secondary metabolite biosynthesis; myricetin involved in flavonoid biosynthesis, flavone and flavonol biosynthesis, and secondary metabolite biosynthesis; and naringenin, cianidanol, and chalconaringenin participating in four pathways including flavonoid biosynthesis, secondary metabolite biosynthesis, phenylpropanoid biosynthesis, and metabolic pathways. Apigenin was involved in five pathways: flavonoid biosynthesis, flavone and flavonol biosynthesis, secondary metabolite biosynthesis, phenylpropanoid biosynthesis, and metabolic pathways. Among the 8 metabolites with decreasing trends, only 3 were annotated: quercetin, kaempferol, and luteolin, all participating in four pathways including flavonoid biosynthesis, flavone and flavonol biosynthesis, secondary metabolite biosynthesis, and metabolic pathways.

2.6 Relationship Between Petal Phenotype and Key Metabolite Content in Three Rose Cultivars As rose color transitioned from white to magenta to deep red, metabolites showing increasing (9 types) and decreasing (3 types) trends were successfully annotated to flavonoid metabolic pathways. Correlation analysis was performed between color phenotypic values and the contents of these 12 key metabolites. As shown in Table 4 , significant negative correlations ($P < 0.01$) were observed between lightness (L*) and both redness (a*) and chroma (C*) across different rose cultivars, indicating that increased petal brightness corresponded to lighter, whiter petals. Pinocembrin and myricetin showed significant negative correlations with L* ($P < 0.01$ and $P < 0.05$, respectively), while pinocembrin exhibited significant positive correlations with a* and C* ($P < 0.01$), demonstrating that accumulation of these compounds enhanced color vividness and reduced petal brightness. Eriodictyol, luteolin, and kaempferol displayed significant positive correlations with L* ($P < 0.01$) and significant negative correlations with a* and C* ($P < 0.01$), indicating their accumulation increased petal brightness toward white coloration. Luteolin showed positive correlation with b* and negative correlation with h°, suggesting that increased content shifted color toward yellow. Hue angle h° was significantly negatively correlated with a* and C* ($P < 0.01$), demonstrating that increased redness reduced hue angle, moving color toward red. These results indicate that pinocembrin and myricetin are primary determinants of red coloration, with increased content intensifying redness while reducing lightness. Conversely, eriodictyol, luteolin, and kaempferol are key factors for white coloration, with increased content promoting whiteness. Luteolin's positive correlation with b* identifies it as a primary component for yellow coloration, which significantly correlates with the yellowish undertones observed in 'Bulgarian White Rose' petals.

Discussion and Conclusion

Flavonoid compounds constitute one of the primary pigment components involved in flower color formation, with anthocyanins being essential constituents whose content differences directly affect plant coloration (Zhong, 2012). Previous studies have demonstrated that cyanidin and its derivatives widely contribute to red petals in various plants (Khoo et al., 2017). Li et al. (2019) identified cyanidin-3-O-glucoside as the main anthocyanin in red camellia petals, while research on 30 rhododendron cultivars with different colors revealed the highest cyanidin content in red varieties (Du et al., 2016). These findings collectively indicate that cyanidin represents the primary pigment component for red coloration in plant petals, consistent with our results. Earlier studies have reported cyanidin as the main flavonoid component in Crimson Glory roses, with significantly higher content than other compounds (Jing et al., 2018), and identified cyanidin-3-glucoside as the primary component of red pigment in Kushui roses (Li, 1997; Gong et al., 2019). Our detection of 58 flavonoid metabolites across the three rose varieties identified only one anthocyanin—cyanidin-3-O-glucoside—accounting for 30.45% of total flavonoids. In Crimson Glory and Kushui roses, cyanidin-3-O-glucoside represented 47.75% and 15.55% of total flavonoids, respectively, far exceeding the 0.04% observed in Bulgarian White Rose. These results demonstrate the crucial role of cyanidin in red petal coloration, establishing cyanidin-3-O-glucoside as the primary substance responsible for red coloration in both Kushui and Crimson Glory roses.

To further investigate differential metabolites underlying distinct rose color formation, extensive research has confirmed that flower color development is influenced by pigment types and contents (Shi et al., 2020; Han et al., 2020). White-purple branch roses contain only flavonoids, whereas pink-flowered 'Pink Purple Branch' and purple-flowered 'Purple Branch' roses contain both flavonoids and anthocyanins (Zhang, 2015). The highest flavonoid compounds in white camellia cultivars 'Silver White Charles' and 'White Phoenix' were luteolin and quercetin-3-O-glucoside (Li et al., 2019), while white roses and chrysanthemums contain only light yellow or nearly colorless flavones and flavonols (Zhou et al., 2009). These conclusions align with our findings. Our screening based on $P < 0.05$ and $VIP \geq 1$ identified 12 key metabolites: 6 flavones, 2 flavanols, 2 flavonols, 1 dihydroflavone, and 1 chalcone. Among these, flavone compounds pinocembrin and myricetin accumulation affected L*, a*, and C* values, with higher content producing more vivid red coloration. In contrast, flavone luteolin, dihydroflavone eriodictyol, and flavonol kaempferol accumulation also influenced L*, a*, and C* values, but with higher content promoting brighter, whiter petals. Luteolin's positive correlation with b* indicates its role in yellow coloration, making it the primary component for yellow pigmentation and significantly correlating with the yellowish undertones in Bulgarian White Rose petals. Therefore, pinocembrin, myricetin, luteolin, eriodictyol, and kaempferol all represent key metabolites influencing rose coloration.

In summary, significant differences in flavonoid metabolite types and contents were detected among the three differently colored roses—'Kushui Rose', 'Crimson Glory', and 'Bulgarian White Rose'—greatly influencing their coloration. The results demonstrate that 58 flavonoid metabolites were detected across the three varieties, with cyanidin-3-O-glucoside, pinocembrin, and myricetin serving as the primary pigment components for red coloration in Kushui and Crimson

Glory roses, while eriodictyol, luteolin, and kaempferol represent the main substances responsible for white coloration in Bulgarian White Rose.

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