Analysis of Flavonoid Metabolism during the Process of Petal Discoloration in Three Malus Crabapple Cultivars
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ABSTRACT: Malus crabapple has high ornamental and ecological value. Here, the flavonoids in the petals of three pink Malus crabapple cultivars, Malus 'Strawberry Parfait' (GD), M. 'Pink Spire' (FY), and M. 'Hongyi' (HY), at the bud stage (flower buds are swollen, and the pistils and stamens are about to appear; L), full bloom stage (the flowers are fully open, and the stigma and anthers have recently appeared; S), and end bloom stage (the stigma and anthers are dry; M) were identified, and their abundances were determined. First, Kodak Color Control Patches were used to describe the colors of petals, and a colorimeter was used to determine the phenotypic values of flower colors. Flavonoids were determined using ultraperformance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS).

In all three crabapple cultivars, the red and yellow hues of the petals gradually disappeared, the color of the flowers changed from bright to dull, and the petals gradually faded. The extent of fading of the red hue of the petals was highest in GD, followed by FY and HY. A total of 302 metabolites were detected in the three cultivars. The content of total flavonoids in the three cultivars significantly differed, but there were no significant differences among species. The total flavonoid content of the three crabapple varieties was highest in HY, followed by FY and GD. The content of the anthocyanins delphinidin-3-O-sophoricoside-5-O-glucoside, pelargonidin-3-O-(6″-O-malonyl)glucoside, pelargonidin-3-O-glucoside, peonidin-3-O-glucoside, and cyanidin-3-O-arabinoside decreased significantly, which resulted in the discoloration of GD petals from L to M. The flavonoids and flavonols in FY might interact with anthocyanins in metabolic pathways. The content of these five anthocyanins decreased slowly, which resulted in the weaker discoloration of FY and HY compared with GD. The content of the five anthocyanins in HY did not decrease significantly, but the content of chalcone increased significantly, which might facilitate the production of anthocyanin auxiliary pigments and result in less pronounced fading of the petals. Cyanidin-3-O-arabinoside and pelargonidin-3-O-glucoside were the key flavonoids of the three crabapple cultivars. The total content and changes in anthocyanins were the key factors affecting petal color development and fading. Nonanthocyanin polyphenols, such as flavonoids, flavonols, and chalcone, are auxiliary pigments that affect petal fading. Overall, the results of this study provide new insights into the mechanism underlying the fading of the color of Malus crabapple flowers, and these new insights could aid the breeding of cultivars with different flower colors.

1. INTRODUCTION
Crabapples refer to members of the genus Malus Mill. in the family Maloideae (Malus Mill.) and Chaenomeles with high ornamental and ecological value. They are popular landscape plants because of their graceful form and aesthetically pleasing colors. During the flowering period of M. 'Strawberry Parfait' (GD), M. 'Pink Spire' (FY), and M. 'Hongyi' (HY), the petals fade from dark pink to light pink, and the magnitude of the fading varies. The fading of floral colors is a common phenomenon in ornamental plants, including Malus hupehensis (Pamp.), Malus spp., Lycoris longituba and Lycoris radiata Herb., and Silene littorea and Silene littorea Brot. The petals of lotus (Nelumbo nucifera) appear to fade from red on the first day of flowering to nearly white on the third day of flowering. In the petals of Chrysanthemum morifolium and Cleome hassleriana, the heavy pink coloration is most pronounced before flower opening, and then the color gradually changes to light pink. The petal color of Paeonia 'Coral Sunset' and 'Pink Hawaiian Coral' fades from coral to light pink and then to pale yellow. Previous studies have shown that this quality trait is mainly determined by the metabolite composition of ornamental plants.
The color of plants is determined by pigments, and there are four natural pigments: flavonoids, carotenoids, chlorophylls, and alkaloids. Flavonoids comprise six main groups of pigments: chalcones, anthocyanidins, flavanones, isoflavonoids, flavones, and flavonols. Chalcone affects the color of yellow flowers; it is also an important substrate for the synthesis of other flavonoids. Flavanoids and flavones are generally light yellow or colorless. Anthocyanins are the main substances responsible for the red, purple, and blue colors of many petals. Several researchers have reported that the content and concentrations of anthocyanins play key roles in flower color formation.

Previous studies have shown that anthocyanins are key pigmentation molecules in *Malus* crabapple petals; differences in flower color among *Malus* crabapple species are caused by differences in their anthocyanins. The fading of the flower color of most plants stems from the inhibition of anthocyanin synthesis; the anthocyanin biosynthetic pathway is highly conserved and has been well studied, and some fading is caused by the degradation of anthocyanins. The accumulation of anthocyanins is the main factor that causes the color of *Hydrangea macrophylla* cv. "Forever Summer" to change from yellow-green to bright blue-violet; anthocyanin accumulation also enhances the redness of petals in *Begonia × hibernalis*. Decreases in the anthocyanin content cause the flower color of *Phalaenopsis* orchids to change from dark purple to pink, which causes lilac petals to gradually change from red-purple to violet, and finally fade to nearly white. *Malus* crabapple is a wild apple plant resource that is rich in flavonoids; its value as a model organism in research is much higher compared with that of cultivated apple plants. Anthocyanins are the main pigments in flavonoid substances. Consequently, most studies of these plants have focused on anthocyanins; fewer studies have examined other components of flavonoids by comparison. The effect of other components on the color of *Malus* crabapples is also important yet understudied.

Studies of *Malus* crabapple within and outside of China have mainly focused on examining variation in the pigment components of crabapple. Few studies have examined the discoloration process of *Malus* crabapple petals. Maintaining the color of *Malus* crabapple and prolonging the flowering period are some of the main focuses of current research on natural pigments. However, no systematic research has been conducted to date. Here, eight-year-old crabapple trees were used to study the fading mechanism of pink crabapple trees; our findings can be used to enhance the ornamental quality of these trees. Specifically, we used ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS), the internal Metware database (MWDDB) of Maiwei Metabolism Company, and public databases with metabolite information to qualitatively and quantitatively analyze the flavonoids in the petals of three *Malus* crabapple cultivars at different flowering stages. We also analyzed the chemical basis underlying the petal discoloration process by characterizing metabolomic differences in flavonoids. The results of this study provide new insights into the mechanism underlying the fading of the color of *Malus* crabapple flowers, and these new insights could be used to obtain new germplasm resources of crabapple and aid the breeding of cultivars with different flower colors.

### Table 1. Dynamic Changes of Flowers Color of GD

| Stage         | L*        | a*        | b*        | C*        | h         |
|---------------|-----------|-----------|-----------|-----------|-----------|
| Bud stage     | 35.29 ± 0.312e | 27.39 ± 0.504a | 10.80 ± 0.217a | 29.190 ± 0.541a | 21.087 ± 0.231a |
| Full bloom    | 58.993 ± 0.494b | 13.100 ± 1.125d | 2.306 ± 0.081c | 13.303 ± 0.627d | 10.170 ± 0.820c |
| End flower    | 65.113 ± 0.332a | 6.013 ± 0.202e | 0.830 ± 0.035d | 6.070 ± 0.182e | 7.909 ± 0.828b |

“Note: The chroma C* and the hue angle h are calculated according to the formulas $C^* = (a^2 + b^2)^{1/2}$ and $h = \arctan(b^*/a^*)$, respectively. The C* value represents the vertical distance to the $L^*$ axis, and greater distances indicate greater chroma. Different lowercase letters in the same column and the same period indicate significant differences between different treatments ($P < 0.05$). Same below.

### Table 2. Dynamic Changes of Flowers Color of FY

| Stage         | L*        | a*        | b*        | C*        | h         |
|---------------|-----------|-----------|-----------|-----------|-----------|
| Bud stage     | 41.801 ± 0.212a | 23.420 ± 0.524a | 0.000 ± 0.020a | 23.560 ± 0.524a | −0.003 ± 0.046a |
| Full bloom    | 50.903 ± 0.508d | 21.186 ± 0.551b | −3.185 ± 0.094d | 21.458 ± 0.557b | −20.36 ± 0.086d |
| End flower    | 54.653 ± 0.602d | 13.467 ± 0.143c | −4.682 ± 0.009e | 13.960 ± 0.134c | −18.785 ± 0.240 |

### Table 3. Dynamic Changes of Flowers Color of HY

| Stage         | L*        | a*        | b*        | C*        | h         |
|---------------|-----------|-----------|-----------|-----------|-----------|
| Bud stage     | 34.786 ± 0.629e | 22.010 ± 0.538a | 2.613 ± 0.008a | 22.166 ± 0.536a | 6.810 ± 0.170a |
| Full bloom    | 46.126 ± 0.173b | 19.480 ± 0.688b | −1.883 ± 0.029d | 20.236 ± 0.781a | −5.380 ± 0.276d |
| End flower    | 52.343 ± 0.514a | 17.620 ± 0.464c | −2.150 ± 0.023d | 17.750 ± 0.464b | −7.000 ± 0.120e |

2. RESULTS

2.1. Color Analysis of Petals. Tables 1, 2, and 3 show the color difference of GD, FY, and HY petals at different flowering and developmental stages. The $L^*$ value gradually increased in all three crabapple cultivars from the bud stage to the end flowering stage, indicating that the brightness increased during the development of the petals. The $a^*$ value gradually decreased, and the magnitude of the decrease in GD, FY, and HY was 75.13%, 42.49%, and 19.95%, respectively, indicating that the red hue of the petals gradually decreased during petal development. The $b^*$ value gradually decreased, indicating that the yellow hue gradually decreased, and the blue color gradually increased. The $C^*$ value gradually decreased, indicating that the petals gradually changed from bright to dim. The results indicated that the red and yellow hue of the petals of GD, FY, and HY gradually decreased, the flower color changed from bright to dull, the petals gradually faded, and the degree of fading at the end of flowering was highest in GD, followed by FY and HY.
2.2. Metabolite Composition and Content. The metabolites of the samples were identified using the targeted metabolome technology of the UPLS-MS/MS platform. The results based on the self-built database and multivariate statistical analysis are shown in Figure 1a; a total of 302 metabolites were detected in the samples of the three crabapple cultivars in the three periods, including 101 flavonols (33.40%), 86 flavonoids (28.50%), 23 chalcones (7.60%), 21 anthocyanins (7.00%), 17 flavanols (5.60%), 11 dihydroflavonols (3.60%), 11 tannins (3.60%), 10 dihydroflavones (3.30%), nine proanthocyanidins (3.00%), seven isoflavones (2.3%), three flavonoid carbonosides (1.00%), two sinensetins (0.60%), and one dihydroisoflavone (0.30%). The content of metabolites in the petals of the three crabapple cultivars was highest in HY, followed by FY and GD. The total content of metabolites in L was greater than that in S and M, and the color was darkest in L, followed by S and M. Among the significantly different metabolites, the total content was highest in HY, followed by FY and GD, and the content of GD was much lower than that of HY and FY. GD was lightest in color, which stemmed from the high M content; it also showed the most significant fading.

2.3. Principal Component Analysis (PCA) and Orthogonal Partial Least-Squares Discriminant Analysis (OPLS-DA) in Different Flowering Periods. The two-dimensional and three-dimensional plots of the PCA scores of the mass spectrometry data of each group of samples and quality control samples are shown in Figure 1b and c, which reveals variation among and within GDL, GDS, GDM, FYL, FYS, FYM, HYL, HYM, and mix.
HYS, and HYM. The amount of variance explained by the first, second, and third principal components was 36.05%, 29.77%, and 10.76%, respectively. There were clear differences among varieties, and the distances between the same varieties among periods were similar. The degree of separation between the three varieties was greater than that between the same variety in the three periods. The differences among L, S, and M for GD and FY were greater than those between S and M. The differences among L, S, and M for HY were smaller than the differences between S and M, which indicates that the accumulation of flavonoids in different varieties of Begonia also differed.

FY and HY samples for each period were close to 1, and that of the GD samples for each period was between 0.65 and 1; the \( |r| \) of each sample was close to 1 (Figure 1d). The type and content of flavonoids of GD varied substantially among periods. The OPLS-DA model revealed that all pairwise comparisons among the three cultivars and three periods were significant (\( P < 0.05 \)), indicating that the results of the model were robust.

Figure 2. Metabolite volcano maps of petals in three different periods of Malus crabapple. (a–c) Metabolite volcano maps of petals in three different periods of GD; (d–f) metabolite volcano maps of petals in three different periods of FY; (g–i) metabolite volcano maps of petals in three different periods of HY. Note: Each point in the volcano plot represents a metabolite, and the abscissa represents the logarithm of the quantitative fold difference of a metabolite in two samples. The ordinate represents the VIP value. Green dots in the figure represent downregulated differentially expressed metabolites. Red dots represent upregulated differentially expressed metabolites, and gray dots represent metabolites detected but not significantly different.
Figure 3. Venn diagrams of differential metabolite numbers in the petals of GD, FY, and HY in three different periods. (a) Differential metabolite numbers of GD. (b) Differential metabolite numbers of FY. (c) Differential metabolite numbers of HY. (d) Differential metabolite numbers of L. (e) Differential metabolite numbers of S. (f) Differential metabolite numbers of M.

Table 4. Different Cumulative Metabolites among Different Comparison Groups of GD, FY, and HY

| compounds                     | class                                   | fold change     | VIP          | P-value       | type |
|-------------------------------|-----------------------------------------|-----------------|--------------|---------------|------|
|                               |                                         | L-vs-S          | S-vs-M       | L-vs-M        | L-vs-S | S-vs-M | L-vs-M | type |
| GD chrysin                    | flavonoid                               | 5.18 2.88       | 14.93 1.29   | 1.36 1.18     | 1.29   | 0.21   | 0.12   | up   |
| 5,7-dihydroxy-2′-methoxy-3,4′-methyleneoxy-dihydroisoflavone | dihydroisoflavone | 14.07 2.60 | 36.60 1.23 | 1.48 1.18 | 1.23 | 0.02   | 0.09   | up   |
| quercetin-3,3′-dimethyl ether | flavonoids                              | 181.63 2.25     | 408.80 1.28  | 1.39 1.22     | 1.28   | 0.05   | 0.06   | up   |
| phloretin-4′-O-(6″-galloyl)glucosidetp-coumaroyl | chalcones                              | 3.99 2.47       | 9.86 1.26    | 1.62 1.21     | 1.26   | 0.02   | 0.00   | up   |
| 3,7-di-O-methylquercetin      | flavonoids                              | 265.80 4.56     | 1213.08 1.29 | 1.60 1.22     | 1.29   | 0.02   | 0.03   | up   |
| 3-O-acetylpimobanksim         | dihydroflavonol                         | 21.82 2.43      | 52.94 1.27   | 1.35 1.20     | 1.27   | 0.01   | 0.19   | up   |
| galangin (3,5,7-trihydroxyflavone one) | flavonoid                               | 131.01 2.67     | 350.21 1.29  | 1.33 1.21     | 1.29   | 0.02   | 0.18   | up   |
| pinocembrin (dihydrochaysm)   | flavonoid                               | 21.48 2.37      | 50.92 1.28   | 1.3 1.20      | 1.28   | 0.02   | 0.17   | up   |
| selagin                        | flavonoid                               | 15.83 2.74      | 43.34 1.03   | 1.00 1.03     | 1.03   | 0.13   | 0.33   | up   |
| leucocyanidin                 | dihydroflavonol                         | 0.43 0.36       | 0.16 1.26    | 1.51 1.19     | 1.26   | 0.01   | 0.01   | down |
| delphinidin-3-O-sophoroside-5-O-glucoside | anthocyanins | 0.27 0.42     | 0.11 1.26   | 1.54 1.20     | 1.26   | 0.02   | 0.02   | down |
| hovertichoside C              | sinensetin                              | 0.32 0.45       | 0.15 1.26    | 1.57 1.21     | 1.26   | 0.01   | 0.02   | down |
| aromadendrin (dihydrokaempferol) | dihydroflavonol | 0.19 0.22     | 0.04 1.21   | 1.24 1.03     | 1.21   | 0.00   | 0.08   | down |
| pelargonidin-3-O-(6″-O-malonyl) | anthocyanins | 0.47 0.47     | 0.22 1.17   | 1.45 1.19     | 1.22   | 0.01   | 0.06   | down |
| glucoside                     | anthocyanins                            | 0.37 0.34       | 0.13 1.27    | 1.61 1.21     | 1.27   | 0.00   | 0.00   | down |
| pelargonidin-3-O-glucoside    | anthocyanins                            | 0.27 0.40       | 0.11 1.25    | 1.62 1.21     | 1.25   | 0.01   | 0.03   | down |
| peonidin-3-O-glucoside        | anthocyanins                            | 0.48 0.50       | 0.24 1.26    | 1.54 1.20     | 1.24   | 0.01   | 0.00   | down |
| cyanidin-3-O-arabinoside      | anthocyanins                            | 0.38 0.35       | 0.13 1.26    | 1.61 1.21     | 1.26   | 0.00   | 0.01   | down |
| trihydroxyflavone-O-glucoside(iso3) | flavonoids     | 5.86 3.02      | 17.72 1.10 | 1.56 1.05 | 1.10   | 0.02   | 0.01   | up   |
| FY 6-methoxyquercetin-3-O-xylside | flavonoids     | 26.5 2.06      | 5.44 1.39 | 1.48 1.29 | 1.39   | 0.05   | 0.01   | up   |
| pentalhydroxymonomethoxyflavone-O-hexosides (iso6) | flavonoids     | 3.97 2.66      | 8.97 1.35 | 1.29 1.43 | 1.35   | 0.07   | 0.07   | up   |
| luteolin-7-O-glucuronide-(2→1)-glucuronide | flavonoids     | 2.28 0.19      | 0.42 1.39 | 1.55 1.30 | 1.39   | 0.02   | 0.01   | up   |
| HY sudachitin                 | flavonoid                               | 2.10 0.24       | 0.50 1.42    | 1.54 1.37     | 1.42   | 0.02   | 0.01   | 0.10   | up   |
| okanin 4′-(6″-O-acetylglucoside) | chalcones     | 3.97 2.66      | 8.97 1.35 | 1.29 1.43 | 1.35   | 0.07   | 0.07   | up   |
| limocitrin-3,7-di-O-glucoside | flavonoid                               | 2.28 0.19       | 0.42 1.39    | 1.55 1.30     | 1.39   | 0.02   | 0.01   | up   |
2.4. Screening of Differential Metabolites in Petals. 

Figure 2a–i shows the volcano plot of the differential metabolites in the petals of GD, FY, and HY during the three different periods. The volcano plot revealed differences in the expression levels and the number of upregulated and downregulated metabolites in the samples of each comparison group. The number of upregulated and downregulated metabolites from L to M was highest in GD, followed by FY and HY. There was a decrease in the total content of significantly different metabolites in GD; the magnitude of the decrease in the content of metabolites, and thus the degree of fading, was larger in GD. The decrease in the content of metabolites was more gradual in FY, and the degree of fading was less pronounced. In HY, the content of metabolites first decreased and then increased. The total content of metabolites in M was lower than that in L. The degree of fading was lowest in M, and the petals in M were still pink.

A Venn diagram of the differential metabolites is shown in Figure 3a–f. Analysis of the differential metabolite screening results for each comparison group revealed three overlapping differential metabolites. There were 67, 68, and 47 common differential metabolites in the petals in L, S, and M, respectively. There were three, three, and 18 common differential metabolites in different periods in FY, HY, and GD, respectively. There were no significant changes in other polyphenols.

The petal discoloration process in FY, GD, and HY was further clarified based on the FC, VIP, and P values (Table 4).

There were three differential metabolites in FY petals, including two flavonoids and one flavanol. Both 6-methoxyquercetin-3-O-xyloside and pentahydroxymonomethoxyflavone-O-hexoside (iso6) were upregulated, and 6-methoxyquercetin-3-O-xyloside was the most significantly different metabolite among the comparison groups. The expression of luteolin-7-O-glucuronide \((2 \rightarrow 1)\)-glucuronide was downregulated, and the decrease in its expression was significant in the three control groups.

There were three differential metabolites in the 3 comparison groups during the discoloration process of HY petals. The expression of 2 flavonoids, 1 chalcone, and 3 metabolites was upregulated. The most upregulated metabolite was sudachitin, and its expression was upregulated 3.97, 2.26, and 8.97-fold in the HYL_vs_HYS, HYS_vs_HYM, and HYL_vs_HYM comparison groups, respectively.

There were 18 differential metabolites across all three comparison groups, including five flavonoids, one dihydroisoflavone, three dihydroflavonols, two flavonols, one chalcone, five anthocyanins, and one sinensetin. Among these, the expression of four flavonoids, one dihydroisoflavone, one dihydroflavonol, two flavonols, and one chalcone was upregulated, and the expression of one flavonoid, two dihydroflavonols, five anthocyanins, and one sinensetin was downregulated. Among the five differential flavonoid metabolites, galangin \((3,5,7\)-trihydroxyflavone) accounted for a large proportion, and the expression of trihydroxyflavone-O-glucoside (iso3) decreased significantly. The expression of the dihydroisoflavonoid 5,7-
metabolites was observed in GD from L to M, followed by FY cyanidin-3-O-arabinoside was downregulated 0.48, 0.50, and regulated 0.27, 0.4, and 0.11-fold; and the expression of pelargonidin-3-O-glucoside was downregulated 0.37, 0.34, and 0.24-fold, respectively. The total content of cyanidin-3-O-arabinoside was the highest.

Table 5. Results of Differential Metabolites between Groups

| group      | Ko00941 | Ko00942 | Ko00943 | Ko00944 | Ko01100 | Ko01110 | up | down | differentially accumulated metabolite |
|------------|---------|---------|---------|---------|---------|---------|----|------|----------------------------------------|
| FYL- FYS   | 2       | 1       | 1       | 1       | 1       | 1       | 50 | 47   | 50                                     |
| FYL- FYS   | 5       | 2       | 1       | 1       | 1       | 1       | 5   | 3    | 8                                      |
| GDL- GDS   | 14      | 5       | 1       | 3       | 6       | 9       | 41 | 36   | 77                                     |
| GDS- GDM   | 6       | 3       | 4       | 1       | 3       | 3       | 14 | 27   | 27                                     |
| GDL- GDM   | 14      | 6       | 1       | 3       | 7       | 11      | 52 | 49   | 101                                    |
| HYL- HYS   | 2       | 1       | 1       | 1       | 1       | 1       | 31 | 8    | 39                                     |
| HYS- HYM   | 2       | 1       | 1       | 1       | 1       | 1       | 9  | 43   | 52                                     |
| HYL- HYM   | 4       | 1       | 3       | 1       | 3       | 3       | 28 | 22   | 50                                     |

dihydroxy-2′-methoxy-3′,4-methylenedioxy-dihydroisoflavone and the dihydroflavonol 3-O-acetylpinobanksin was significantly upregulated in the GDL_vs_GDS, GDS_vs_GDM, and GDL_vs_GDM comparison groups, and the expression of the two dihydroflavonols leucocyanidin and aromadendrin (dihydrokaempferol) was also significantly downregulated. Both flavonoids accounted for a large proportion of the total metabolites, and the expression of 3,7-di-O-methylquercetin and quercetin-3,3′-dimethyl ether decreased and then increased. The expression of the chalcone phloretin-4′-O-(6′-galloyl)-glucoside-coumaroyl was significantly increased. The expression of sinensetin hovertichoside C significantly decreased. The expression of the five anthocyanins significantly decreased. In L, S, and M, the expression of delphinidin-3-O-sophoroside-5-O-glucoside was downregulated 0.27, 0.42, and 0.11-fold; the expression of pelargonidin-3-O-(6′-O-malonyl)glucoside was downregulated 0.47, 0.47, and 0.22-fold; the expression of pelargonidin-3-O-glucoside was downregulated 0.37, 0.34, and 0.13-fold; the expression of peonidin-3-O-glucoside was downregulated 0.27, 0.4, and 0.11-fold; and the expression of cyanidin-3-O-arabinoside was downregulated 0.48, 0.50, and 0.24-fold, respectively. The total content of cyanidin-3-O-arabinoside was the highest.

Anthocyanins are important substances determining the color of plants. Dynamic changes in the five significant differentially expressed anthocyanins in the three flowering periods in crabapple are shown in Figure 4a. The anthocyanin content in L was highest in GD, followed by HY and FY. The color of GD was the darkest. The expression of anthocyanins was downregulated, and the magnitude of the downregulation from L to M was highest in GD, followed by FY and HY, indicating that the significant declines in these five anthocyanins were the determinants of the magnitude of fading, as the degree of fading was consistent with these anthocyanin declines.

2.5. KEGG Enrichment Analysis of Differential Metabolites. The differential metabolites were annotated and displayed using the KEGG database. The differential metabolites might be involved in two or even multiple metabolic pathways (Table 5). A total of 101, 58, and 50 differential metabolites were screened from L to M in GD, FY, and HY. The number of differential metabolites from L to M (52 upregulated and 49 downregulated) was highest in GD among the three crabapple varieties, and this was consistent with the differential metabolites indicated in the volcano plot. Figure 4b–d show the degree of enrichment of the differential metabolites and the overall changes in the differential metabolites involved in different metabolic pathways. The differential metabolites of FY, GD, and HY were mainly involved in the flavonoid biosynthesis pathway (Ko00941), and the highest number of differential metabolites was observed in GD from L to M, followed by FY and HY. Overall, differential metabolites tended to be upregulated in FY, and these metabolites were enriched in the metabolic pathway and secondary metabolism synthesis pathway (Ko01110). There were several differential metabolites in GD that were significantly enriched in the anthocyanin biosynthetic pathway (Ko00942), and the expression of the differential metabolites involved in the anthocyanin biosynthetic pathway were generally downregulated. Differential metabolites in HY were significantly enriched in the flavonoid and flavonol biosynthesis (Ko00944) pathways, and the expression of the differential metabolites was upregulated. The flavonoid biosynthesis pathway, metabolic pathway, secondary metabolism synthesis pathway, and dynamic changes in differential metabolites in the anthocyanin biosynthesis pathway appear to be the causes of the petal fading of the three crabapple cultivars, and the degree of fading was most pronounced in GD, followed by FY and HY.

3. DISCUSSION

The instrument color measurement method quantifies flower color, which can increase the accuracy of the data; it is widely used to measure the flower color of horticultural plants, such as Freesia hybridra30 and Camellia japonica.31 From the bud stage to the end of the flowering stage, the red and yellow hues of the petals gradually decreased, the color of the flower changed from bright to dull, and the petals gradually faded. The degree of fading of the red hue of the petals was highest in GD, followed by FY and HY. This indicates that the fading of Begonia petals is characterized by the weakening of red and yellow hues, and the degree of weakening is positively correlated with the degree of fading.

In this study, the total content of flavonoids significantly differed among varieties; the most pronounced difference observed was in the total pigment content, and changes in the pigment content were correlated with color depth. The flavonoid content of the three crabapple varieties was highest in HY, followed by FY and GD, and the total flavonoid content of GD was much lower than that of HY and FY; the color depth in the S and M periods was highest in HY, followed by FY and GD. Some researchers have studied three herbaceous peony cultivars and suggested that the total content of flavonoids is not the only factor affecting the color of plants.32 In our study, the chalcone phloretin-4′-O-(6′-cinnamoyl)glucoside was the most abundant in GD and lowest in HY and FY. However, the color of the petals was lightest in GD, and the most significant fading was observed in S to M. Thus, color changes appear to be related to flavonoid type, and the contribution of different flavonoids to petal coloration varies. The flavonoid composition of the three cultivars of crabapple was basically the same, and this is consistent with a previous study of Paeonia delavayi showing that...
the composition of flavonoids in petals of six different colors was the same and that changes in colors stemmed from differences in the content of flavonoids. The total metabolites of flavonoids and flavonols made up a relatively large proportion of all metabolites, and there were no significant differences in the composition of flavonoids and flavonols among the different cultivars. There is thought to be no direct relationship between the coloration and fading of petals. *Rosa rugosa* petals accumulated a large number of flavonoids without forming any anthocyanins, but they might interact with anthocyanins and affect flower color expression, resulting in yellow petals. However, this result is inconsistent with the findings of Saito et al. showing that the composition of flavonoids is not correlated with petal color and hue. Yang et al. suggested that the dramatic decrease in the anthocyanin content during the flowering process of the peony was the main reason for the change in flower color from red to yellow. A PCA of petal color in different periods revealed differences in the accumulation of flavonoids in different crabapple cultivars. The OPLS-DA model was used to make pairwise comparisons of the three cultivars and three periods, and all pairwise comparisons were significant (P < 0.05), indicating that the results of the model were robust. These findings are consistent with those of Gao et al. demonstrating significant differences in flavonoids in different parts of *Kadsura coccinea*.

Zhang et al. found that anthocyanins determine changes in color from red to purple in plants, and Xia et al. found that cyanidin glycosides were the main substances underlying changes in color from red to purple in *Rhododendron pulchrum* petals. Zhang et al. demonstrated that cyanidin-3-O-galactoside, pelargonidin-3-O-galactogluicoside, and cyanidin-3-O-succinic acid-arabinoside affect the redness of *Chamaemelus speciosa*. In this study, the total content of cyanidin-3-O-arabinoside and pelargonid-3-O-glucoside contributed the most to the total content of flavonoids, which was lowest in GD, and the color of GD during flowering was lighter compared with that of the other two cultivars. The shallow color indicates that the total content of cyanidin and pelargonid has a major effect on the color of petals, which is consistent with the results of Tanaka et al. During the blooming process in the three crabapple cultivars, the flower color faded from dark pink to pink or even pinkish-white, and similar changes have been observed in other plants. Li et al. showed that the reduction of the anthocyanin content in Brunfelsia acuminate caused the petals to change from dark purple to white; Zhang et al. suggested that the pink cultivars of crabapple faded uniformly from purple to red-pink–light pink (or nearly white). In this study, the expression of delphinidin-3-O-sophoricside-5-O-glucoside, geranium-3-O-(6′-O-malonyl)glucoside, geranium-3-O-glucose, paeonolfrorin-3-O-glucoside, and cyanidin-3-O-arabinoside was significantly downregulated from L to M, and the content of these anthocyanins was higher in L than in S and M. The significant downregulation of these five anthocyanins might be the determinant of fading; however, one flavonoid and two flavonols were significantly upregulated from L to M in GD, and the interaction of these compounds with anthocyanins might affect the color of flowers. In FY petals, the expression of 6-methoxyquercetin-3-O-xylidoside and pentahydroxymonome-thoxyflavone-H-oxepoxide (iso6) was significantly upregulated, and the expression of luteolin-7-O-glucuronide-(2′→1)-glucuronide was significantly downregulated; these findings are inconsistent with the changes in the pigments during petal opening documented in Li et al. The synthesis of noncoloring substances might cause the petals to fade, but the actual cause of the observed changes requires clarification using gene expression analyses. Yin et al. found that chalcones can generate dihydroflavones through chalcone isomerase and then generate various types of flavanones and flavonoids to promote the accumulation of anthocyanins and flavonoids; they can also be converted into colorless naringenin to promote the formation of anthocyanins, flavonoids, and flavonols. The content of chalcones in HY petals was upregulated during flowering, suggesting that chalcones play an important role in petal fading as auxiliary pigments during the flowering of HY. During the flowering process, the content of anthocyanins decreased significantly, which caused the color of GD petals to change from dark pink to pinkish-white or even white, and the anthocyanin content of FY and HY decreased slowly, which resulted in less pronounced fading of the color of the petals. These two cultivars did not fade to white until the end of flowering. With the exception of the significant differential metabolites, the expression of other polyphenols such as proanthocyanidins did not change significantly in each period, which might stem from the transformation of materials during the growth and development of petals. KEGG analysis revealed that the dynamic changes in differential metabolites involved in multiple metabolic pathways such as the anthocyanin biosynthesis pathway caused the petals of the three crabapple cultivars to fade. Flavonoids are generated from phenylalanine through the phenylpropanoid pathway, and phenylalanine is synthesized via the shikimate pathway. In this pathway, the amino acid phenylalanine is used to synthesize 4-coumarin-CoA, which can combine with malonyl-CoA to generate chalcones; it can then undergo a series of enzymatic modifications to synthesize flavanones, followed by dihydroflavonols and anthocyanins. Zhu and Lu suggested that almost every intermediate substrate in the anthocyanin metabolic pathway can be directed to other flavonoid branches, and variation in any structural gene can alter the flow direction of the substrate. This can affect various steps in the anthocyanin synthesis pathway, and the final synthetic anthocyanin aglycone species are key factors determining the final coloration of plants. The transformation of red-purple varieties to pale varieties might be caused by various mechanisms. Han et al. suggested that the fading of *M. hupheniasis* stemmed from the inhibition of anthocyanin biosynthesis by corresponding structural genes. Yang et al. found that anthocyanin degradation rates varied during the flowering process of peony, which causes the flower color to gradually change from red to orange to yellow, and Xu et al. suggested that anthocyanins in petals are usually only degraded and not synthesized after the blooming period. The degree of fading of the three cultivars of crabapple varied. The total content of cyanidin-3-O-arabinoside decreased by 76%, 22%, and 11% in GD, FY, and HY, respectively, and the total content of pelargonidin-3-O-glucoside decreased by 87%, 15%, and 19% in GD, FY, and HY, respectively, and the total content of flavanols decreased by 19% in GD, FY, and HY, respectively. The most significant fading in GD might stem from the degradation of anthocyanins in the anthocyanin biometabolism pathway, and the fading of FY and HY was less pronounced, which might stem from a reduction in the degradation of anthocyanins and the effect of regulatory factors in the flavonoid metabolic pathway. Anthocyanins and flavonoids might also be competing substrates; however, additional experiments are needed to confirm this hypothesis.
4. CONCLUSIONS

The total content of cyanidin-3-O-arabinoside and pelargonidin-3-O-glucoside determines the pink color of the petals of the three crabapple cultivars.

Table 6. Sample Number Information

| tissue site                      | sample name | group |
|----------------------------------|-------------|-------|
| Malus 'Strawberry Parfait' bud stage | GDL-1       | GDL   |
|                                  | GDL-2       |       |
|                                  | GDL-3       |       |
| Malus 'Strawberry Parfait' full bloom stage | GDS-1       | GDS   |
|                                  | GDS-2       |       |
|                                  | GDS-3       |       |
| Malus 'Strawberry Parfait' end bloom stage | GDM-1       | GDM   |
|                                  | GDM-2       |       |
|                                  | GDM-3       |       |
| Malus 'Pink Spire' bud stage     | FYL-1       | FYL   |
|                                  | FYL-2       |       |
|                                  | FYL-3       |       |
| Malus 'Pink Spire' full bloom stage | FYS-1       | FYS   |
|                                  | FYS-2       |       |
|                                  | FYS-3       |       |
| Malus 'Pink Spire' end bloom stage | FYM-1       | FYM   |
|                                  | FYM-2       |       |
|                                  | FYM-3       |       |
| Malus 'Hongyi' bud stage         | HYL-1       | HYL   |
|                                  | HYL-2       |       |
|                                  | HYL-3       |       |
| Malus 'Hongyi' full bloom stage  | HYS-1       | HYS   |
|                                  | HYS-2       |       |
|                                  | HYS-3       |       |
| Malus 'Hongyi' end bloom stage   | HYM-1       | HYM   |
|                                  | HYM-2       |       |
|                                  | HYM-3       |       |

Changes in the content of five anthocyanins during flowering had a major effect on petal fading. In the anthocyanin biometabolism pathway, the degradation of anthocyanins resulted in the most significant fading of GD, and the slow decline in anthocyanins resulted in the reduced fading of HY and FY.

Significant changes in one flavonoid and two flavonols in FY might interact with anthocyanins to mitigate the fading of petals. The content of chalcones in the petals of HY was significantly upregulated during the flowering period. Chalcones might provide substrates for the synthesis of various flavanones and flavonoids during the flowering period of HY and act as auxiliary pigments for anthocyanins, thereby reducing the magnitude of fading.

5. MATERIALS AND METHODS

5.1. Experimental Materials. Three eight-year-old pink crabapple varieties similar in size and growth potential, *Malus 'Strawberry Parfait', M. 'Pink Spire', and M. 'Hongyi', in Begonia Garden in Mazhuang Town, were used in experiments. The central flowers of the outer branches were collected at three stages: the flowering stage, the full blooming period, and the end flowering period. Three grams of flower tissue was removed, wrapped in tin foil, and stored in a −80 °C refrigerator for identification of flavonoids. Kodak Color Control Panels were used to describe the color. The sample names and pictures showing the flowering status are shown in Table 6. Figure 5a shows GD. Figure 5b shows FY, and Figure 5c shows HY.

5.2. Determination of Petal Color. After the petals were collected, some petals were used for measurements of flower color with a CM-5 spectrophotometer (NF333 spectrophotometer, Nippon Denshokk, light source C/2°) according to the International Commission on Illumination (CIE) colorimetric system.30

5.3. Flavonoid Metabolite Preparation and Extraction. In this study, ultraperformance liquid chromatography—tandem mass spectrometry (UPLC—MS/MS, UPLC, SHIMADZU Nexera X2, www.shimadzu.com.cn/; MS, Applied Biosystems 4500 Q TRAP, www.appliedbiosystems.com.cn/) from Maiwei Metabolism Company (Wuhan Metwell Biotechnology Company, Wuhan City, Hubei Province, China) was used for the primary metabolomics analysis. The sample extraction process and analytical conditions were as follows. (1) Biological samples were freeze-dried using a vacuum freeze-dryer (Scientz-100F). (2) The freeze-dried samples were crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. (3) A total of 100 mg of lyophilized powder was dissolved with 1.2 mL of 70% methanol solution, followed by vortexing for 30 s every 30 min for a total of six cycles; the samples were then placed into a refrigerator at 4 °C overnight. (4) Following centrifugation at 12 000 rpm for 10 min, the extracts were filtrated (SCAA-104, 0.22-μm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/) before UPLC—MS/MS analysis.

An Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm) was used. Mobile phase A and mobile phase B were ultrapure water and acetonitrile, respectively (both phases A and B contained 0.1% acetic acid). The column temperature was 40 °C, the flow rate was set to 0.35 mL/min, and the injection volume was 4 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.51

5.4. Metabolomic Data Analysis. The sample extracts were mixed and used as a quality control (QC) sample for analyzing the repeatability of our method. One QC sample was analyzed after every 10 test samples during analysis. Principal component analysis (PCA), cluster analysis, orthogonal partial

![Figure 5](https://doi.org/10.1021/acsomega.2c03820)
least-squares discriminant analysis (OPLS-DA), and other multivariate statistical analyses were used to reduce the dimensionality of the multidimensional data to establish a reliable mathematical model that summarizes the characteristics of the metabolic spectrum. Differential metabolites among samples were identified using fold change (FC) from univariate analysis and variable importance in projection (VIP) from the OPLS-DA model thresholds: FC ≥ 2 or FC ≤ 0.5 and VIP ≥ 1. This method considers differences in metabolites during the classification and discrimination of each group of samples in the model. The selected differential metabolites were annotated using the KEGG database.52

Analyst 1.6.1 software was used to process the MS data and perform qualitative and quantitative MS analysis on the metabolites of each sample using the local metabolic database. The primary and secondary spectral data were qualitatively analyzed using the internal Metware database (MWDB) of Maiwei Metabolism Company and a public database containing metabolite information. Metabolite quantification was performed using triple-quadrupole mass spectrometry in MRM mode.53

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Notes
The authors declare no competing financial interest.

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