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Correlation between the composition and content of anthocyanins and leaf coloration in *Nekemias grossedentata*

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Abstract

Background *Nekemias grossedentata* is the raw material for processing vine tea. Purple vine tea holds market potential for diversifying products, yet the mechanism underlying purple leaf formation remains unclear. This study aimed to elucidate the relationship between anthocyanin profiles and leaf coloration in this species.

Results Green leaves (LM1, LM2) exhibited higher total chlorophyll content (2.011–2.218 mg/g). Anthocyanins were identified as the dominant pigments for color, with total content in purple leaves (ZM1, ZM2, ZM3; 2322.31–7493.18 µg/g) significantly exceeding that in green leaves (166.11–1014.05 µg/g). A total of 81 anthocyanin components were characterized. Purple leaves primarily contained 32 cyanidin, 10 delphinidin, and 10 peonidin derivatives. Distinct accumulation patterns were observed: ZM1 was enriched with cyanidin-3-O-glucoside (809.87 µg/g), ZM2 with malvidin-3,5-O-diglucoside (1620.16 µg/g), and ZM3 with delphinidin-3,5-O-diglucoside (1884.85 µg/g). OPLS-DA analysis identified cyanidin-3-O-glucoside, delphinidin-3-O-galactoside, delphinidin-3,5-O-diglucoside, and petunidin-3-O-glucoside as key differential compounds. Correlation analysis revealed that the a^* value is critical for hue distinction (positive in purple/reddish leaves, negative in green leaves). Total anthocyanin content showed a highly significant negative correlation with the L^* value (lightness), while peonidin derivatives displayed a highly significant positive correlation with the a^* value, identifying them as a key driver for purple coloration.

Conclusions This study clarifies the correlation between specific anthocyanins and color parameters in *N. grossedentata* leaves. The findings provide new insights into the biochemical mechanism of leaf color variation and offer a theoretical foundation for breeding new cultivars with diverse and desirable leaf color traits.

Key words *Nekemias grossedentata*, Anthocyanins, Leaf color, Targeted metabolomics, UPLC-MS/MS

Background

Nekemias grossedentata is a perennial woody liana belonging to the Vitaceae family. Its leaves are once or twice pinnately compound with serrated margins, and

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young leaves exhibit germplasm variation in green and purple coloration [1] [2]. The primary distribution of wild *N. grossedentata* includes regions such as Jiangxi, Fujian, Hubei, Hunan, Guangdong, Guangxi, Guizhou, and Yunnan (Flora of China). Since its leaves were approved as a novel food ingredient in 2013, the tender leaves have been used as the core raw material for "Vine Tea" , which has developed into a characteristic industry with an annual output value exceeding 2.7 billion CNY. The cultivation area in Zhangjiajie and Xiangxi Prefecture alone reaches 20.6 thousand hectares [3] [4]. As a functional tea, Vine Tea is exceptionally rich in flavonoids (up to 30%–47.2%), particularly dihydromyricetin, earning it the title "King of Flavonoids" [5]. It possesses significant pharmacological effects, including analgesic, anti-inflammatory, and antioxidant activities [6] [7]. In recent years, the rapid development of the Vine Tea industry has led to increasingly prominent product homogeneity. However, the discovery of purple-leaf mutants from wild *N. grossedentata* resources and their subsequent processing into "Purple Vine Tea" has opened a new pathway for product innovation and value enhancement.

Plant organ coloration is predominantly regulated by carotenoids, flavonoids (particularly anthocyanins), chlorophylls, and betalains. Anthocyanins, belonging to the flavonoid class of secondary metabolites, are crucial water-soluble pigments widely accumulated in vascular plants. They are key compounds responsible for red, blue, and purple hues in plants **Error! Unknown switch argument.Error! Unknown switch argument..** The six major types of anthocyanidins (aglycones) are cyanidin (Cy), delphinidin (Dp), pelargonidin (Pg), peonidin (Pn), petunidin (Pt), and malvidin (Mv). Among these, Cy is the most widely distributed, while Mv has the highest degree of methylation [10][11]. Anthocyanin accumulation is closely linked to color formation. Cy derivatives often appear reddish-purple, whereas Dp and Mv derivatives tend towards bluish-purple [12]-[14]. Cyanidin-3-O-glucoside and peonidin-3-O-glucoside are key pigments for red and purple coloration, respectively, with their content and ratio directly influencing color intensity and hue [15] [16]. Beyond regulating plant colors, anthocyanins possess potent antioxidant, immunomodulatory, and metabolic intervention functions. These include scavenging hydroxyl radicals, inhibiting the NF- κ B/MAPK signaling pathway, and activating the AMPK pathway [17]-[20]. Consequently, they hold broad application prospects in the fields of functional foods and medicine.

Studies have shown that pH affects the stability and color manifestation of anthocyanins: anthocyanins appear red at pH < 7, purple at pH 7–8, and turn blue at pH > 11 [21] [22]. The modification of anthocyanidin aglycones determines hue variations; for example, Cy exhibits a reddish-purple hue, Dp tends towards bluish-purple, while methylated derivatives such as Pn and Mv enhance the stability of the purple-red color [23]. Leaf colorimetric values influence anthocyanin stability; in roses and jujubes, the a^* value is often positively correlated with anthocyanin content, while the L^* value is mostly negatively correlated with chlorophyll content [16]-[24]. In recent years, with the deepening of anthocyanin research, the expression patterns of regulatory genes (e.g., MYB, bHLH) and structural genes have been dynamically analyzed via transcriptomics, and the accumulation patterns of

anthocyanins such as cyanidin and pelargonidin have been quantified via metabolomics. This has enabled the construction of gene-metabolite-phenotype causal relationships, elucidating the molecular basis of leaf color variation in different species under various environmental conditions. For instance, cyanidin-3-O-glucoside and peonidin-3-O-glucoside have been identified as the main components responsible for the purple leaf color in ornamental plants like *Triadica sebifera* and *Acer pseudosieboldianum* [25]-[26]. However, research on the coloration mechanism of anthocyanins in the leaves and fruits of dual-purpose medicinal and edible plants is limited to a few fruit trees. *N. grossedentata*, as a tea-like plant with prominent medicinal value, has long been studied in academia for the analysis of flavonoid components like dihydromyricetin and their pharmacological activities. The mechanism underlying leaf color change and anthocyanin biosynthesis in its leaves has not yet been explored.

Based on preliminary resource collection and evaluation, this study utilized wild purple and green *N. grossedentata* materials to systematically measure leaf color parameters, photosynthetic pigment content, and total flavonoid content. The composition and enrichment pathways of anthocyanins were further analyzed using UPLC-MS/MS. The aims of this study were to: (1) elucidate the phenotypic and fundamental physiological and biochemical characteristics of *N. grossedentata* with different leaf colors; (2) identify the key anthocyanin components responsible for the purple phenotype; and (3) analyze the quantitative relationships between anthocyanin accumulation and leaf color parameters. The findings will provide data to support the understanding of the coloration mechanism in *N. grossedentata* and guide targeted breeding efforts.

Materials and Methods

Plant Materials

Green germplasms (LM1, LM2) and purple germplasms (ZM1, ZM2, ZM3) of *N. grossedentata* (Hand.-Mazz.) J. Wen & Z. L. Nie were cultivated in a greenhouse at Hunan Gengxin Nongyuan Technology Co., Ltd. (29°28' N, 110°33' E) and identified by Professor Weiqiang Qin of Jishou University. For subsequent measurements, plants with good and uniform growth status were randomly selected from each germplasm. Each material was sampled in three independent replicates (with 3 biological replicates per sampling), resulting in a total of 9 independent sample sets. The sample information is presented in Table 1.

Table 1 Sample Information

Code	Altitude(m,LAT)	Longitude(°E,LON)	Latitude(°N,LAT)	Location
LM1	664.20	109.82	29.20	Zhangjiajie, Hunan
LM1	490.00	109.85	29.00	Yongshun, Hunan
ZM1	213.20-235.20	114.27	25.37	Dayu, Jiangxi
ZM2	229.00	111.48	28.90	Changde, Hunan
ZM3	484.10-506.10	108.77	27.81	Taiping, Guizhou

Instruments and Equipment

AF224C analytical balance (0.1 mg accuracy, Shanghai Hengping Scientific

Instrument Co., Ltd., China); EX125DZH analytical balance (0.01 mg accuracy, Ohaus Instruments Changzhou Co., Ltd., China); DSA300-GL1 ultrasonic cleaner (Fuzhou Desen Precision Co., Ltd., China); CenLee18K high-speed centrifuge (Xiangli Scientific Instrument Co., Ltd., Hunan, China); DS-100 chroma meter; UV757CRT UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., USA); Synergy HTX multi-mode microplate reader (BioTek Instruments, Inc., USA); Lei-Ci PHS-3C pH meter (Shanghai Yidian Scientific Instrument Co., Ltd., China).

Determination of Leaf Color Indices

Leaf color parameters were measured using a spectrophotometric colorimeter. The brightness (L_1), red-green value (a_1), and yellow-blue value (b_1) of the leaf adaxial side, as well as the corresponding parameters (L_2 , a_2 , b_2) of the abaxial side, were recorded. In the colorimetric system, higher L^* values indicate lighter colors, positive a^* values indicate redness while negative values indicate greenness, and positive b^* values indicate yellowness while negative values indicate blueness.

Determination of Physiological and Biochemical Indices

Leaf pH Fresh leaf samples (0.50 g) were washed, dried, ground into homogenate, mixed with 30 mL distilled water, and shaken for 6 min. The mixture was filtered, and the pH of the filtrate was measured using a pH meter.

Chlorophyll Content Fresh leaf tissue (0.20 g) was cut into pieces, ground with 95% ethanol and quartz sand until the tissue turned white, then filtered and made up to 25 mL with 95% ethanol. Using 95% ethanol as blank, absorbance was measured at 665, 649, and 470 nm. The concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car) were calculated according to standard formulas and converted to content per unit fresh weight (mg/g).

Total Anthocyanin Content The total anthocyanin content was determined by the pH differential method following the instructions of the Plant Total Anthocyanin Assay Kit (Jiangsu Jingmei Biotechnology Co., Ltd., China). Absorbance was measured at 530 nm and 700 nm, and the total anthocyanin content was calculated accordingly.

Anthocyanin Component Analysis

Sample Preparation Leaf samples were flash-frozen in liquid nitrogen and stored at -80°C . 50 mg of vacuum freeze-dried powder was weighed, and 500 μL of extraction solution (50% methanol aqueous solution containing 0.1% hydrochloric acid) was added. The mixture was vortexed for 10 min, then centrifuged at 13,400 rpm (4°C) for 3 min. The supernatants were combined and filtered through a 0.22 μm membrane prior to analysis.

UPLC-MS/MS Analysis Chromatographic conditions: An Acquity BEH C18 column (1.7 μm , 2.1 mm $\times$ 100 mm) was used. The mobile phase consisted of (A) 0.5% formic acid in water and (B) 5% formic acid in methanol, with gradient elution. The flow rate was 0.35 mL/min, and the column temperature was 40°C . Mass spectrometric conditions: Electrospray ionization in positive mode (ESI^+) was applied, with an ion source temperature of 550°C and a voltage of 5500 V. Scanning was

performed in multiple reaction monitoring (MRM) mode, and quantification was carried out based on standard curves.

Screening of Differential Metabolites Orthogonal partial least squares-discriminant analysis (OPLS-DA) was employed to screen differential metabolites, using thresholds of $VIP > 1$ and $FC \geq 2$ (or ≤ 0.5). The significance of the differential metabolites was further verified by ANOVA ($P < 0.05$).

Statistical Analysis

Mean values and standard deviations were calculated using Excel 2020. Statistical significance of differences (one-way ANOVA, Duncan's multiple range test) and Pearson correlation analysis (significance level $p < 0.05$) were performed with SPSS Statistics 22.0. Untargeted metabolomics data were subjected to Log_2 transformation, and multivariate statistical analysis (PCA and OPLS-DA) was carried out using SIMCA-P 13.0. All experiments were performed with three biological replicates, and data are presented as mean $\pm$ standard deviation.

Results

Comparative Analysis of Leaf Color Phenotypic Traits in *N. grossedentata*

Leaf color parameters serve as crucial indicators for evaluating the color characteristics of plant leaves. Their variation is often closely associated with pigment composition and metabolite accumulation. The a^* value (red-green axis) is a key parameter for distinguishing different leaf colors. As shown in **Table 2**, the a^* values of the five germplasms differed significantly. The a_2 values (abaxial side) of the purple germplasms (ZM1, ZM2, ZM3) were positive (reaching 8.78 ± 3.36 in ZM2), whereas those of the green germplasms (LM1, LM2) were negative (approximately -6.77), indicating that leaves of the purple germplasms appeared reddish, which is highly consistent with anthocyanin accumulation. The lightness parameter (L value) of the green germplasm leaves was consistently higher than that of the purple germplasms; a higher L^* value corresponds to a brighter leaf appearance. The b^* value (yellow-blue axis) also varied regularly among germplasms: the green germplasms exhibited relatively high b^* values (e.g., 30.51 ± 2.41 in LM1), indicative of a yellowish hue, whereas the purple germplasms showed lower b^* values (e.g., 15.58 ± 3.54 in ZM1), which may reflect changes in the proportion of carotenoids or chlorophylls. Hence, leaf color diversity is primarily governed by the a^* value for red-green hue transition, modulated by the L^* value for lightness, and supplemented by the b^* value for yellow-blue contribution. Together, these three parameters define a continuous phenotypic spectrum from green to purple, providing a clear phenotypic basis for elucidating the regulatory mechanisms of anthocyanin metabolism.

Table 2 Leaf color phenotypic characteristics in *N. grossedentata*

Code	L_1	a_1	b_1	L_2	a_2	b_2
LM1	45.57 ± 1.24^a	-9.53 ± 0.60^c	30.51 ± 2.41^a	50.18 ± 1.90^a	-6.77 ± 1.37^c	21.71 ± 1.58^a
LM2	45.74 ± 1.14^a	-8.66 ± 0.70^c	27.54 ± 2.26^b	51.28 ± 2.35^a	-6.75 ± 1.21^c	20.55 ± 1.85^a

Code	L ₁	a ₁	b ₁	L ₂	a ₂	b ₂
ZM1	37.37±1.83 ^b	-5.65±3.00 ^b	15.58±3.54 ^c	40.27±5.44 ^b	1.68±6.20 ^b	11.42±5.09 ^b
ZM2	38.35±2.22 ^b	-4.51±3.30 ^a	20.50±2.45 ^c	35.76±2.46 ^c	8.78±3.36 ^a	7.30±4.35 ^c
ZM3	37.87±2.89 ^b	-8.53±1.72 ^b	24.85±2.18 ^d	37.00±3.56 ^c	8.18±4.45 ^a	7.35±3.92 ^c

Different lowercase letters indicate a significant difference within the column ($p < 0.05$).

Analysis of Photosynthetic Pigment and Total Anthocyanin Accumulation in *N. grossedentata*

Photosynthetic Pigments

Photosynthetic pigments are key substances for plant photosynthesis, and chlorophyll content directly determines the greenness of leaves. The accumulation of photosynthetic pigments exhibited regular differences among germplasms with different leaf colors. As shown in Fig. 1, the contents of Chl a, Chl b, and Car in the green germplasms (LM1, LM2) were significantly higher than those in the purple germplasms (ZM1, ZM2, ZM3). Specifically, the green germplasms accumulated higher levels of Chl a, with contents of 1.53 and 1.20 mg/g in LM1 and LM2, respectively. Among the purple germplasms, the Chl a content of ZM1 was 0.75 mg/g, while that of ZM2 and ZM3 was ≤ 0.65 mg/g, showing significant differences among groups ($p < 0.05$). The accumulation of Chl b followed a similar pattern, with higher levels in the green germplasms than in the purple ones. Car also showed a consistent trend, with contents of 0.33 and 0.20 mg/g in LM1 and LM2, respectively, while Car contents in all purple germplasms were ≤ 0.20 mg/g. These results indicate that the transition from green to purple leaf color is accompanied by a decrease in photosynthetic pigment content, which may be due to regulatory or antagonistic effects of anthocyanin accumulation on the synthesis or stability of photosynthetic pigments.

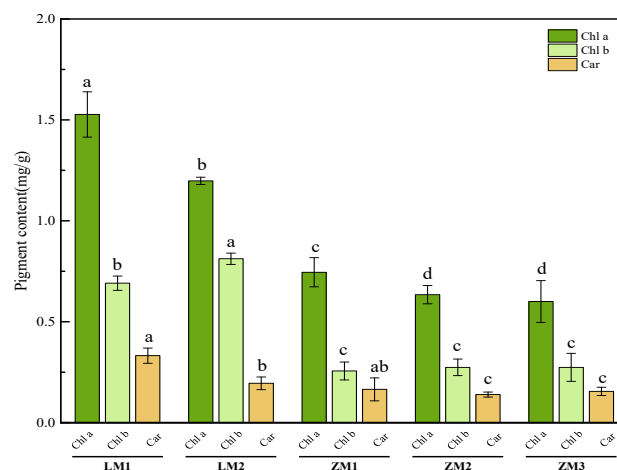


Fig. 1 Photosynthetic pigment contents in *N. grossedentata* with different leaf colors

Different letters indicate significant differences for the same index among different materials ($p < 0.05$). The same applies below.

Analysis of Total Anthocyanin Content

Anthocyanins are the core metabolites responsible for the red and purple hues in plant leaves. As shown in Fig. 2, the accumulation of total anthocyanins (quantified as cyanidin-3-O-glucoside equivalents) differed extremely significantly among the *N. grossedentata* leaves. The content in the purple germplasms was substantially higher than that in the green germplasms, further corroborating the phenotypic observations of leaf color. The accumulation was most pronounced in ZM1, reaching a high level of 936.04 $\mu\text{g/g}$, which was significantly greater than that in ZM2 and ZM3. Among the green germplasms, the content in LM1 was slightly higher than in LM2, but both remained at low levels. LM2 contained 101.97 $\mu\text{g/g}$, which was only 10.9% of the ZM1 content. This indicates that the intensity of anthocyanin accumulation is a key biochemical indicator distinguishing purple from green leaves. Furthermore, the variation in total anthocyanin content aligned with the trend of the leaf color parameter a^* value, providing additional evidence for the dominant role of this pigment in leaf coloration.

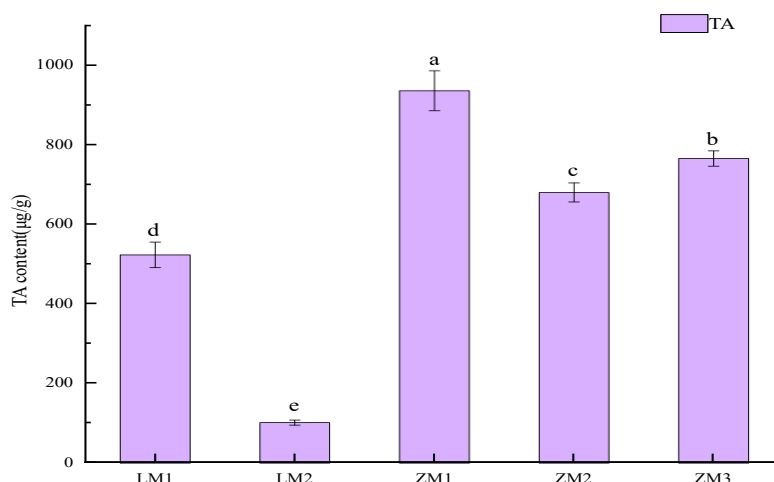


Fig. 2 Comparison of total anthocyanin content in *N. grossedentata* materials with different

Comparison of Leaf pH in *N. grossedentata*

Leaf cellular pH is a key environmental factor influencing the coloration and stability of anthocyanins. As shown in Fig. 3, the leaf pH values differed significantly among the *N. grossedentata* germplasms with different leaf colors. The leaf pH of the purple germplasms was significantly lower than that of the green germplasms, corresponding to the trend of substantial anthocyanin accumulation in the former. Specifically, the pH values of LM1 and LM2 were relatively high, at 5.73 and 5.76, respectively. In contrast, the pH of ZM1 was 5.49, while those of ZM2 and ZM3 were 5.21 and 5.08, respectively, with significant differences among groups ($p < 0.05$). This indicates that the transition from green to purple leaf color is accompanied by a decrease in leaf cellular pH, suggesting that a lower intracellular pH environment may be more conducive to the stable accumulation and vivid coloration of anthocyanins. Together, these physiological and biochemical factors determine the final coloration phenotype

of the leaves.leaf colors

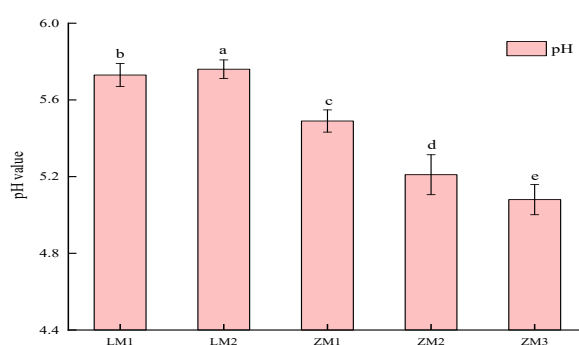


Fig. 3 pH in *N. grossedentata* materials with different leaf colors

Correlation between Leaf Color Phenotype and Pigment Accumulation

The formation of leaf color in *N. grossedentata* results from a coordinated interplay between the trade-off of anthocyanin and photosynthetic pigment accumulation, along with changes in the photophysical properties of the leaves. As shown in Fig. 4, correlation analysis revealed that anthocyanin content was extremely significantly positively correlated with leaf redness (a^* value; $r = 0.394$, $p < 0.01$), directly driving the shift towards reddish and purple hues. Concurrently, it showed a significant negative correlation with leaf lightness (L^* value; $r = -0.710$, $p < 0.01$), resulting in the darker coloration of purple leaves. Furthermore, anthocyanin accumulation was significantly correlated with a lower cellular pH ($r = -0.592$, $p < 0.01$), providing a necessary microenvironment for the stable storage of the pigments. In summary, the continuous spectrum of leaf colors from green to purple is fundamentally determined by the combined effects of anthocyanin-dominated coloration, the concomitant reduction of photosynthetic pigments, and the overall adjustment of the leaf's optical properties.

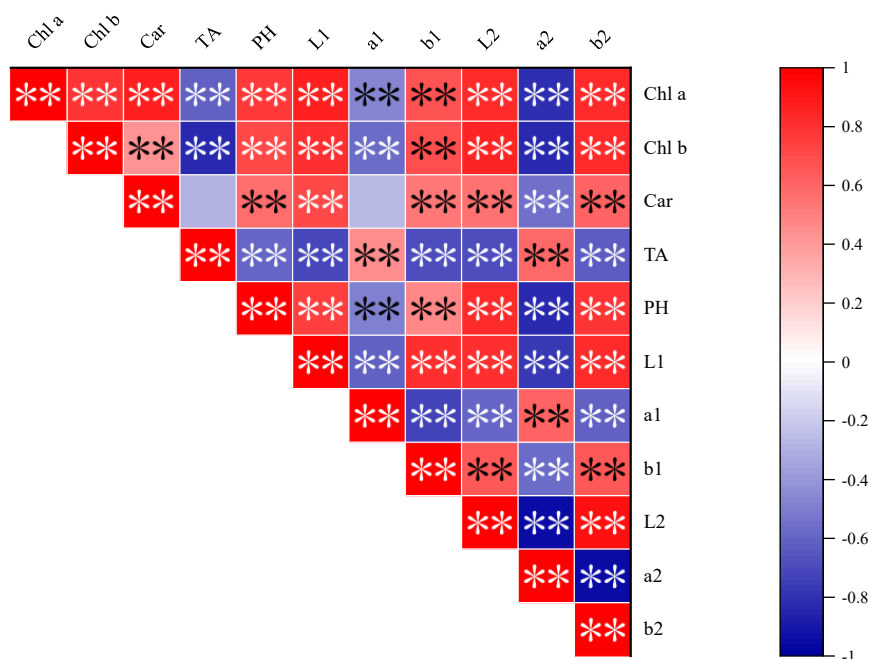


Fig. 4 Correlation of key indicators associated with leaf color formation in *N. grossedentata*

* $p < 0.05$, ** $p < 0.01$

Analysis of Anthocyanin Content and Composition in *N. grossedentata*

Targeted metabolomics analysis revealed that *N. grossedentata* leaves contain 81 anthocyanin-related compounds, categorized into eight major types: Cy, Pg, Dp, Pn, Mv, proanthocyanidins, Pt, and flavonoids. Among these, Cy derivatives were the most abundant, comprising 32 components (39.5%) and forming the primary structural framework of the anthocyanin profile. The other types followed in order: Pg (11 components, 13.6%), Dp and Pn (10 components each, 12.3%), Mv (7 components, 8.6%), proanthocyanidins (6 components, 7.4%), Pt (3 components, 3.7%), and flavonoids (2 components, 2.5%), with Pt and flavonoids being the least represented. Regarding the number of components, the difference between purple and green germplasms was not pronounced. For instance, ZM2 contained 56 components, ZM3 had 46, ZM1 had 49, while LM1 and LM2 also contained 48 and 48 components, respectively. However, the key determinant of leaf color phenotype is not the diversity of components but rather the total accumulation of anthocyanins. As shown in **Fig. 5**, the anthocyanin accumulation level in purple germplasms was significantly higher than that in green germplasms. Specifically, ZM2 exhibited the highest total content (7448.87 $\mu\text{g/g}$), followed by ZM3 (3414.67 $\mu\text{g/g}$) and ZM1 (2322.31 $\mu\text{g/g}$). In contrast, the total contents in the green germplasms LM1 and LM2 were only 1014.05 $\mu\text{g/g}$ and 166.11 $\mu\text{g/g}$, respectively, with ZM2 being 45 times that of LM2. These results indicate that the primary biochemical factor underlying the difference between purple and green leaf colors is the total amount of anthocyanin accumulation, rather than the number of anthocyanin components.

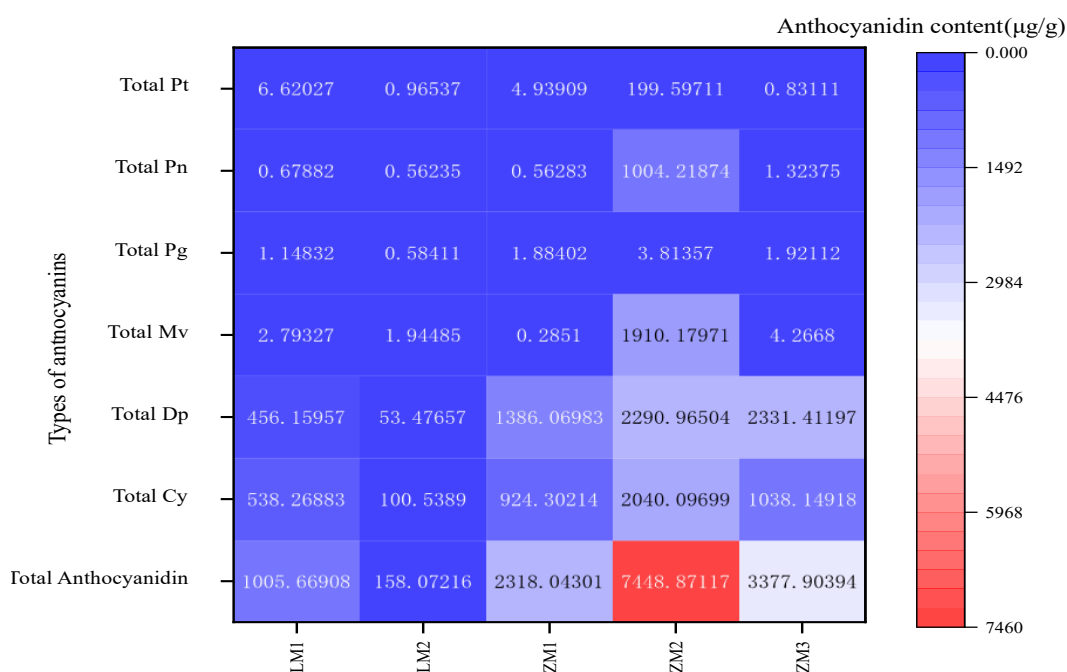


Fig. 5 Anthocyanin composition and content in different materials

The content of the six major anthocyanin categories represents the sum of multiple components within each respective class; "Total anthocyanin content" refers to the sum of all detected components and is distinct from the single indicator "Total anthocyanin glycosides."

As shown in **Fig. 6**, hierarchical cluster analysis (HCA) clearly divided all samples into two major clusters based on the overall similarity of their anthocyanin compositions. Samples with the purple phenotype (ZM2, ZM3, ZM1) formed a distinct cluster, while those with the green phenotype (LM1, LM2) were closely grouped into another cluster. This visualization provides direct evidence for a fundamental metabolomic differentiation underlying the observed leaf color variation. Within the purple cluster, ZM2 and ZM3 were aggregated first, indicating that they possess highly similar metabolic "fingerprints". ZM1, on the other hand, constituted an independent branch under this main cluster, displaying accumulation patterns that are both related to and distinct from ZM2/ZM3. This suggests the potential existence of different chemotypes among the purple germplasms.

Heatmap analysis (**Fig. 5**) corroborated this clustering result and provided a more detailed visualization of metabolite accumulation differences. The purple germplasms exhibited consistently high accumulation for the majority of anthocyanin components, whereas the green germplasms generally showed low-level expression. A more focused examination of the metabolic profiles further differentiated the accumulation patterns among ZM2, ZM3, and ZM1. ZM2 and ZM3, forming a sub-cluster, were more closely related. The former was characterized by high accumulation of methylated derivatives, while the latter was enriched with specific non-methylated diglycosides. ZM1 displayed a distinct accumulation pattern compared to the other two. This cluster structure reflects, at the metabolomic level, the chemical basis of leaf color divergence. It also dissects the potential compositional signatures within the purple phenotype, providing a basis for elucidating differences in their synthesis and modification pathways. Metabolic modification profiles differed significantly among germplasms with different leaf colors. ZM2 was rich in methylated derivatives, such as A44 (malvidin-3,5-O-diglucoside) with a content as high as 1620.164 $\mu\text{g/g}$, and A98 (petunidin-3-O-glucoside) at 199.579 $\mu\text{g/g}$. In contrast, ZM3 showed significant accumulation of non-methylated diglycosides; its A19 (delphinidin-3,5-O-diglucoside) content reached 1884.847 $\mu\text{g/g}$, the highest among all detected components. The anthocyanin profile of ZM1 was relatively balanced, with prominent accumulation of common monoglycosides like A11 (cyanidin-3-O-glucoside) (809.866 $\mu\text{g/g}$). In comparison, the total content in green germplasms was extremely low, containing only basic components, such as trace amounts of A116 (cyanidin-3-xylosyl-galactoside) in LM2, suggesting their biosynthesis or modification pathways might be constrained. These characteristics indicate that the purple germplasms not only form the biochemical foundation for leaf coloration but also constitute a natural repository of high-value anthocyanin components, demonstrating clear application potential in industries related to natural pigments, functional foods, and cosmetic ingredients.

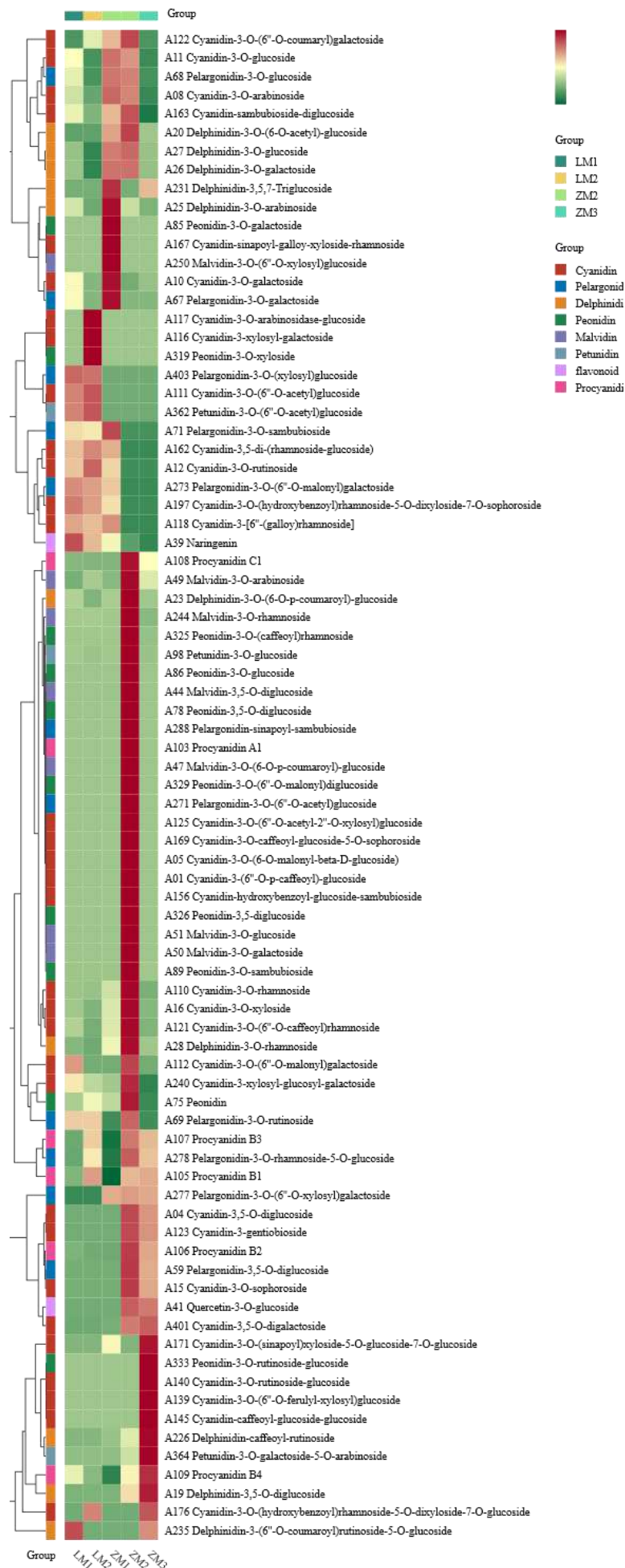


Fig. 6 Heatmap of Anthocyanin Composition Differences Among Five *N. grossedentata* Materials

Screening of Differential Metabolites in *N. grossedentata*

Based on UPLC-MS/MS metabolomics analysis, the leaf color phenotype of *N. grossedentata* is closely associated with its specific anthocyanin accumulation pattern. Using the green-leaved germplasm LM2 as the reference, differential metabolites were screened by comparing it with the high-flavonoid green-leaved germplasm LM1 and the three purple-leaved germplasms (ZM1, ZM2, ZM3), respectively, yielding varying numbers of differential metabolites. As shown in **Fig. 7**, 28 differential metabolites were identified between LM1 vs. LM2, with 23 up-regulated and 5 down-regulated; 39 differential metabolites were found between ZM1 vs. LM2, with 27 up-regulated and 12 down-regulated; 64 differential metabolites were identified between ZM2 vs. LM2, with 49 up-regulated and 15 down-regulated; and 51 differential metabolites were found between ZM3 vs. LM2, with 30 up-regulated and 21 down-regulated. The number of up-regulated metabolites was significantly greater than that of down-regulated metabolites, indicating that the purple leaf phenotype is primarily associated with the extensive activation of anthocyanin biosynthesis pathways.

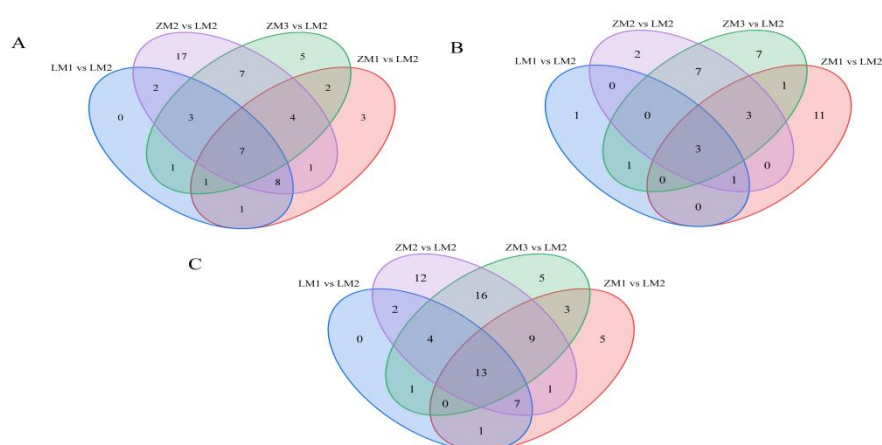


Fig. 7 Overlap of differential metabolites identified in pairwise comparisons between LM2 and the other four *N. grossedentata* samples with varying leaf colors

A:Up-regulated metabolites, B:Down-regulated metabolites, C: Total differential metabolites.

Notably, although the number of differential metabolites varied among the different comparison groups, the up-regulated anthocyanin types were highly overlapping, forming a stable metabolic basis for the purple phenotype. As shown in **Fig. 9**, A11 (cyanidin-3-O-glucoside) exhibited significant accumulation in all comparisons between the purple-leaved germplasms and LM2. Its up-regulation magnitude ($\text{Log}_2\text{FC} > 10$) far exceeded that of other anthocyanins and its expression in the green-leaved germplasm LM1, indicating that it is the most critical marker compound for distinguishing purple/green leaf color and contributing to the deep purple hue. Furthermore, A26 (delphinidin-3-O-galactoside), A19 (delphinidin-3,5-O-diglucoside), A98 (petunidin-3-O-glucoside), and others were also

significantly up-regulated in the purple germplasms, collectively enhancing the coloration effect. The chemical structures of these four key anthocyanins are shown in Fig. 8.

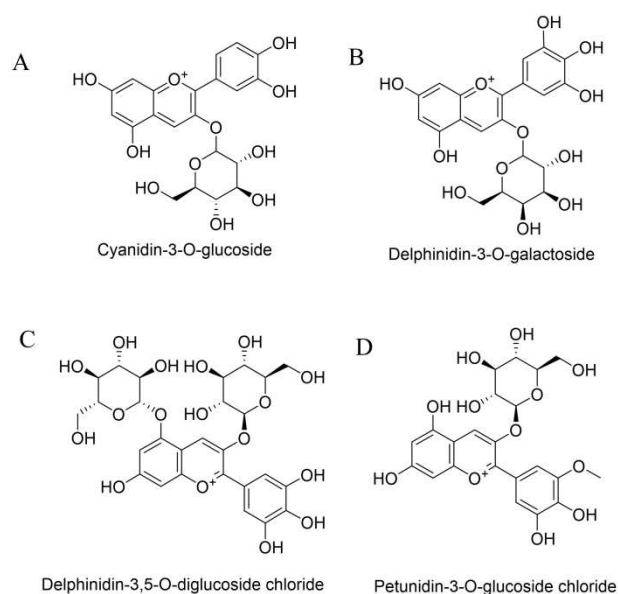


Fig. 8 Key anthocyanin glycosides that were accumulated in the purple leaves of *N. grossedentata*.

However, significant differences exist among the purple germplasms regarding the dominant accumulated anthocyanin types and their chemical modifications, which directly influence the final hue expression. ZM2 leaves were markedly enriched in methylated derivatives represented by malvidin (A50, A51) and petunidin, modifications typically associated with enhanced pigment stability and deep purplish-red tones. In contrast, ZM3 exhibited exceptionally high accumulation of non-methylated diglycosides corresponding to delphinidin and cyanidin (A19, A04), structures that often shift the coloration towards vivid bluish-purple hues. Meanwhile, ZM1 demonstrated a high efficiency in accumulating delphinidin and cyanidin monoglycosides, suggesting an efficient metabolic flux from upstream synthesis to initial glycosylation steps.

In contrast, both green germplasms exhibited a weak anthocyanin biosynthetic pathway, though the underlying mechanisms may differ. Although LM1 showed a detectable basal level of certain anthocyanins, its overall accumulation was far lower than that in the purple germplasms. Considering its background as a high-flavonoid resource, it is speculated that the carbon metabolic flux in LM1 may be primarily directed towards competitive branch pathways, such as those for flavonoids or proanthocyanidins.

In summary, the leaf color of *N. grossedentata* is predominantly governed by the overall intensity of anthocyanin accumulation, while the specific hue differences are determined by the fine-tuned regulation of downstream modification steps, such as methylation and glycosylation. The diversity among purple germplasms results from the specific enhancement of different modification patterns, whereas the green phenotype arises from the suppression of this pathway or the diversion of metabolic

flux. The key differential metabolites identified here, along with their accumulation patterns, provide direct chemical evidence and targets for subsequent research aimed at elucidating the molecular regulatory network underlying leaf color trait formation.

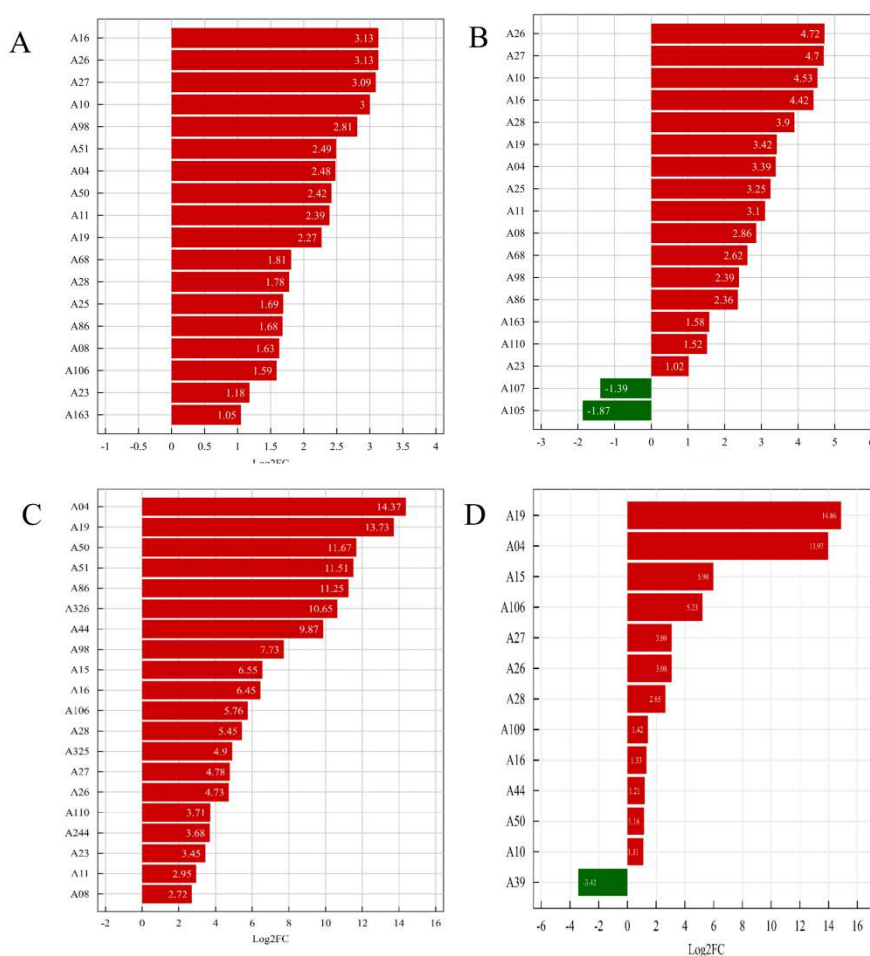


Fig. 9 The expression distribution of differentially expressed metabolites across different comparison groups

The horizontal axis represents the log₂fold change (Log₂(FC)), and the vertical axis represents the differentially expressed metabolites. A, B, C, and D correspond to the comparison groups LM1 vs. LM2, ZM1 vs. LM2, ZM2 vs. LM2, and ZM3 vs. LM2, respectively.

Metabolic Subtyping of Anthocyanin Biosynthesis in *N. grossedentata* Based on KEGG Pathway Analysis

Based on the KEGG pathway enrichment results (Fig. 10), the anthocyanin biosynthesis in *N. grossedentata* germplasms with different leaf colors exhibited significant pathway preferences and metabolic subtypes, which in turn influenced their anthocyanin composition and accumulation levels. In the anthocyanin biosynthesis pathway (ko00942), a total of 32 metabolites were annotated, mainly including cyanidin (Cy), delphinidin (Dp), malvidin (Mv), petunidin (Pt), peonidin (Pn), and their corresponding glycosylated (e.g., glucosides, galactosides) and acylated (e.g., malonylated) derivatives. The accumulation heatmap revealed that most of these derivatized anthocyanins were highly accumulated in the purple

germplasms, while their contents were generally low in the green germplasms. This pattern was further corroborated by the KEGG enrichment analysis of the differential metabolites (Fig. 10). Compared to LM2, LM1 and ZM1 were primarily enriched in pathways such as "Metabolic pathways", "Biosynthesis of secondary metabolites", and "Anthocyanin biosynthesis". In addition to the aforementioned pathways, ZM2 and ZM3 also showed significant enrichment in the "Flavone and flavonol biosynthesis" pathway, indicating the specific activation of these pathways in the purple germplasms.

The metabolic pathway subtypes varied with leaf color. ZM2 was dominated by malvidin derivatives, with malvidin-3,5-O-diglucoside accounting for 16.1% of the total anthocyanins, indicating that its leaf color primarily originates from the methylation modification pathway. This pathway involves multiple methylation reactions of delphinidin catalyzed by O-methyltransferases (OMT). In contrast, ZM3 was characterized by a predominance of non-methylated diglycosides, such as delphinidin-3,5-O-diglucoside (34.4%) and cyanidin-3,5-O-diglucoside (9.7%). Its biosynthetic pathway exhibited high activity at the catalytic steps involving flavonoid 3',5'-hydroxylase (F3'5'H) and downstream UDP-glucose: flavonoid glycosyltransferases (UFGT). ZM1 contained significantly higher levels of monoglycosides, with cyanidin-3-O-glucoside (~35.2%) and delphinidin-3-O-glucoside (~27.4%) being prominent, suggesting that its late-stage modification may be dominated by specific monoglycosyltransferases. In contrast, the total anthocyanin content in the green germplasms was extremely low, and the strength of their biosynthesis pathways was weak.

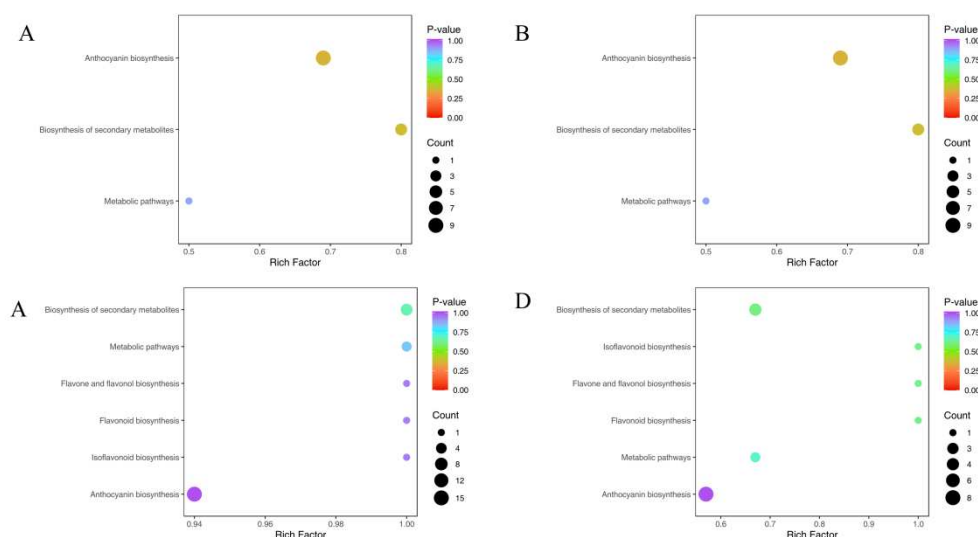


Fig. 10 KEGG enrichment analysis of differential metabolites among different leaf-color comparison groups in *N. grossedentata*

A, B, C, and D represent the comparative results between LM1 vs. LM2, ZM1 vs. ZM2 vs. LM2, and ZM3 vs. LM2.

Correlation Analysis between Leaf Color and Anthocyanin Composition

Through Pearson correlation analysis of leaf color parameters (L, a, b) and the content of various anthocyanins in five *N. grossedentata* germplasms, this study revealed the quantitative relationship between pigment composition and leaf phenotypic traits. As shown in **Fig. 11**, among the individual components, a large number of anthocyanins significantly reduced leaf lightness. Specifically, cyanidin (Cy)-type compounds (e.g., A11, A121, A16, A15) exhibited the highest absolute values of negative correlation coefficients, identifying them as the key components contributing to the darkest leaf coloration. This indicates that higher anthocyanin accumulation leads to darker leaf color and lower lightness. From the correlation analysis results, several compounds showed a strong positive correlation with the a value, the key parameter determining the red-green chromaticity. Among these, peonidin-3-O-glucoside (A86) and malvidin-3,5-O-diglucoside (A44) displayed the most prominent correlation coefficients. Further summary indicates that the a* value was highly significantly positively correlated with methylated anthocyanins, particularly Pn and Mv. This result is mutually corroborated by the phenotypic data: the ZM2 germplasm, which had the highest content of Pn and its derivatives, also achieved the highest a* value. Furthermore, the b* value, representing the yellow-blue chromaticity, showed a highly significant positive correlation with the total anthocyanin content. All types of anthocyanins exhibited a strong positive correlation with the b* value, suggesting that anthocyanin accumulation overall enhanced the yellow tone or weakened the blue tone of the leaves.

In summary, the leaf color phenotype in *N. grossedentata* is shaped by both the overall intensity and the compositional specificity of anthocyanin accumulation. A high total anthocyanin content establishes the color foundation by reducing lightness (L) and influencing the yellow-blue balance (b), whereas the accumulation levels of peonidin (Pn) and malvidin (Mv), represented by specific highly correlated components, directly regulate the critical hue shift from red to purple (a*). This finding quantitatively identifies the key coloring substances, providing clear metabolic targets for the subsequent directional manipulation of leaf color.

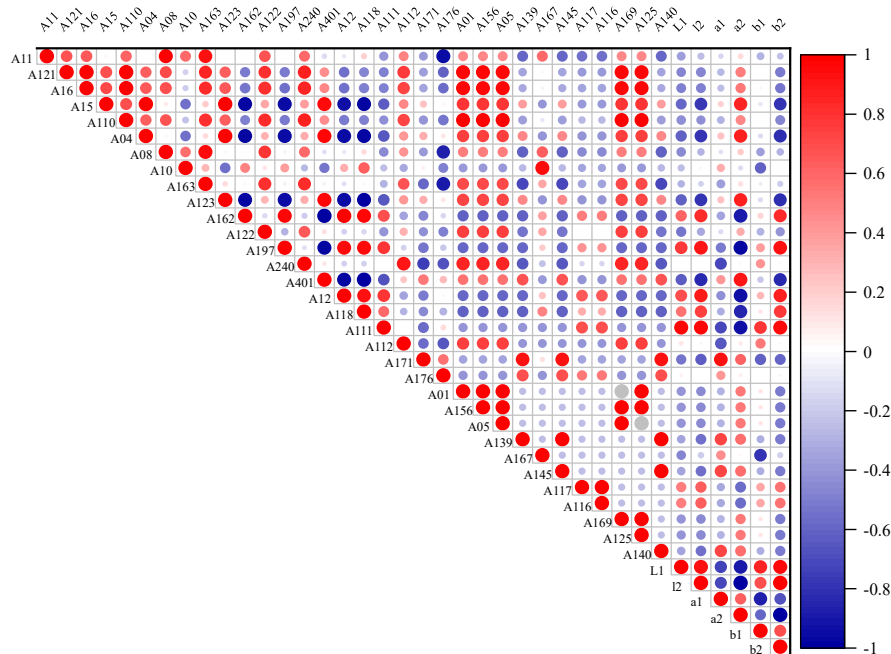


Fig. 11 Heatmap of correlation matrix among leaf color parameters in *N. grossedentata*

Discussion

As a raw material plant for Vine Tea, the diversity of leaf color in *N. grossedentata* is an important indicator for quality-oriented breeding. By integrating leaf color phenotypes, physiological and biochemical indices, and targeted metabolomics data, this study reveals the chemical basis and metabolic regulatory features underlying leaf coloration.

Correlation Between Leaf Color Parameters and Anthocyanin Accumulation

Plant leaf color is co-regulated by the total amount and composition of pigments. First, colorimetric parameters (L, a, b) provide an objective and quantifiable description system [27]. Second, anthocyanins, as key pigments for red and purple hues, directly determine color intensity through their accumulation level [28][30]. This study found that the total anthocyanin content in the purple germplasms of *N. grossedentata* (2322.31–7493.18 $\mu\text{g/g}$) was significantly higher than that in the green germplasms (166.11–1014.05 $\mu\text{g/g}$). The colorimetric parameters showed lower L values and higher a* values in the purple germplasms. This indicates that the total anthocyanin content was highly significantly negatively correlated with leaf lightness (L), and anthocyanin deposition led to tissue darkening, a finding consistent with previous research [24] [16]. The a2 value (abaxial side) proved to be the most sensitive indicator for distinguishing between purple and green leaves (e.g., ZM2 a2 reached 8.78), and redness was highly significantly positively correlated with anthocyanin content, a conclusion corroborated by studies on purple-leaf plum [27]. The reason is that high concentrations of anthocyanins, as dark pigments deposited in leaf cell vacuoles, strongly absorb visible light, leading to a decrease in leaf lightness. Their specific absorption spectra cause an increase in the a value, confirming the

dominant role of anthocyanins in the formation of the red hue. Changes in the b^* value reflect adaptive adjustments in the relative proportions of photosynthetic pigments, such as chlorophylls and carotenoids, under conditions of substantial anthocyanin synthesis. In summary, the total anthocyanin content establishes the material basis for the continuous color variation from green to purple in *N. grossedentata* leaves by significantly reducing lightness (L), dominating the red hue (a), and influencing the yellow-blue tone (b^*).

Regulation of Anthocyanin Coloration by Cellular pH

The final coloration of anthocyanins is highly dependent on the acidity/alkalinity of the cellular vacuolar environment. This study found that the pH of purple leaves in *N. grossedentata* (5.08–5.49) was significantly lower than that of green leaves (5.73–5.76) and was negatively correlated with anthocyanin content. This pattern is similar to anthocyanin accumulation in various other plant species. For instance, vacuolar pH is a key factor affecting petal redness in roses [15], and vacuolar acidification is a prerequisite for the vibrant red color in rose petals, regulated by transcription factors [31]. Phenotypically, plant vacuolar pH can modulate color phenotypes; a lower pH facilitates red coloration in petals, whereas a higher pH can lead to a mottled purple appearance. **Error! Reference source not found.** [22]. From a biochemical perspective, in a slightly acidic environment, anthocyanins primarily exist in the stable flavylium cation form, which appears red/purple. An increase in pH causes their transformation into colorless pseudobase or blue quinonoidal base structures, resulting in fading or a bluish hue [32]. Therefore, the relatively acidic vacuolar environment maintained by the purple germplasms in this study provides optimal conditions for the stability and coloration of the accumulated key anthocyanins, serving as a critical factor for the formation and maintenance of the purple leaf color.

Identification of Key Pigment Substances and Metabolic Subtypes

As the key coloring substance for red and purple hues, the accumulation level of anthocyanins directly determines the intensity of coloration [28] [29]. This study found that the coloration basis of purple leaves in *N. grossedentata* stems from derivatives of peonidin (Pn) and malvidin (Mv). Both these aglycones are products of anthocyanin methylation, a modification that significantly enhances the chemical stability of the pigments and shifts their color towards a deep, stable purplish-red [33]. Methylated monoglycosides, such as peonidin-3-O-glucoside and malvidin-3-O-galactoside, are the most prominent coloring substances, indicating strong activity of O-methyltransferases in the downstream steps of anthocyanin synthesis. This finding aligns more closely with the pattern observed in red rose petals, which also require peonidin accumulation [15], and is consistent with the mechanism in *Camellia oleifera*, where specific anthocyanins dominate red phenotypes [34]. It stands in contrast to the common pattern seen in jujube, *Chimonanthus*, and pineapple, where cyanidin (Cy) or its derivatives are the dominant pigments in different tissues [24] [35] [36]. Extensive research confirms that both the total amount and composition of anthocyanins jointly regulate the final phenotype. For instance, red

leaf coloration in red maple results from the trade-off between chlorophyll degradation and the accumulation of anthocyanins, particularly cyanidin-3,5-O-diglucoside [37]. The deep red color of radish roots is dominated by pelargonidin, while cyanidin and malvidin lead to deep purple roots; such compositional differences are primarily determined by the activity of genes like F3'H[38]. Similarly, in quinoa, the specific accumulation of cyanidin and peonidin derivatives in red inflorescences is supported by the upregulation of specific transporter genes, highlighting the importance of transport processes in determining the final pigment composition [39]. At the molecular level, the accumulation of anthocyanins is under the core regulation of transcription factors such as MYB and bHLH, which control their synthesis by activating structural genes in the anthocyanin biosynthesis pathway **Error! Unknown switch argument. Error! Unknown switch argument., Error! Unknown switch argument..** In summary, *N. grossedentata* exhibits purple leaves by specifically and highly accumulating methylated derivatives of peonidin and malvidin. This not only enriches our understanding of the diversity of plant coloring substances but also provides clues for elucidating the specific metabolic regulatory network (e.g., strong OMT activity) underlying this trait.

KEGG pathway enrichment analysis revealed that the "anthocyanin biosynthesis" pathway was activated in the purple-leaved *N. grossedentata*, which aligns with the metabolic reprogramming patterns observed during red leaf coloration and flower color formation **Error! Unknown switch argument. Error! Unknown switch argument..** The molecular circuit maintaining the green leaf phenotype in tobacco is also characterized by the concurrent upregulation of both chlorophyll and anthocyanin biosynthesis pathways, reflecting a conserved, synergistic regulatory logic in pigment metabolism networks across species [41]. However, significant metabolic subtyping exists among different germplasms, stemming from the differential regulation of downstream modification networks. This is similar to the mechanism in radish, where differences in anthocyanin composition result in varying root colors [38]. Specifically, ZM2 is highly enriched with methylated derivatives of malvidin (Mv) and petunidin (Pt), indicating extremely strong O-methyltransferase (OMT) activity that channels metabolic flux towards the methylation branch, thereby conferring a typical deep purplish-red hue. Such structures endow the pigments with enhanced chemical stability and antioxidant activity, granting them prominent advantages as natural food colorants or functional food ingredients, enabling both long-lasting coloration and concurrent health benefits [42][44]. In contrast, ZM3 accumulates diglycosides of delphinidin (Dp), suggesting that the activities of flavonoid 3',5'-hydroxylase (F3'5'H) and glycosyltransferases (UGTs) are more pronounced, directing metabolic flux towards the highly hydroxylated and diglycosylated branch, which may lead to a bluish-purple tint. Its outstanding radical-scavenging capacity holds application value in photoprotection and anti-aging, making it suitable for skincare or nutraceutical product development [45] [46]. The metabolic profile of ZM1 is dominated by basic monoglycosides, implying a high metabolic flux for this component and ease of further downstream modification, positioning it as a preferred raw material for the scaled production of specific

anthocyanin derivatives [47] [48]. This significant compositional divergence under the same purple phenotype is consistent with the conclusion that specific anthocyanin profiles determine subtle color variations in grape or sweet cherry. It reflects how genetic backgrounds differentially regulate key modifying enzymes (e.g., OMT, F3'5'H, UFGT) and upstream transcription factors (e.g., MYB, bHLH) **Error! Unknown switch argument. Error! Unknown switch argument. Error! Unknown switch argument. Error! Unknown switch argument.**, and may also be subject to fine epigenetic regulation, such as by the long non-coding RNAs targeting anthocyanin synthesis genes identified in potato [50], ultimately shaping the diverse coloration characteristics.

Metabolic Trade-off Between Anthocyanins and Photosynthetic Pigments

The final manifestation of leaf color results from the combined action of different pigments. In this study, the accumulation of anthocyanins in the purple germplasms was accompanied by a significant decrease in the content of photosynthetic pigments. This suggests a potential diversion or trade-off of carbon skeletons and energy resources within the phenylpropanoid metabolic pathway. When genetic signals drive resources towards the anthocyanin biosynthesis pathway, the precursors or energy available for synthesizing photosynthetic pigments such as chlorophyll may be correspondingly reduced. This leads to a fading of the leaf's "green background," thereby accentuating the red and purple hues of anthocyanins. This competitive relationship between pigments is widespread in many ornamental trees, evidenced by a high correlation between the anthocyanin/chlorophyll ratio and leaf color intensity [32]. In *Phoebe bournei*, the red-leaf period is characterized by high anthocyanin and low chlorophyll content, transitioning to decreased anthocyanins and increased chlorophyll as leaves turn green, demonstrating a clear reciprocal relationship. Furthermore, in species like *Prunus virginiana*, the seasonal accumulation of anthocyanins and the degradation of chlorophyll show tight coupling at both physiological and molecular levels [51]. Additionally, soluble sugars serve not only as the carbon skeleton precursors for anthocyanin synthesis but also as important signaling molecules that can activate the expression of key genes in the anthocyanin biosynthesis pathway. Their content is significantly positively correlated with anthocyanin accumulation [52]. Although soluble sugars were not measured in this study, the efficient photosynthesis or sugar transport capacity in the purple germplasms may provide an adequate substrate supply for anthocyanin synthesis.

Conclusions

This study found that the purple leaves of *N. grossedentata* are predominantly colored by methylated derivatives of peonidin (Pn) and malvidin (Mv). The purple coloration results from the overall activation of the anthocyanin biosynthesis pathway and the specific methylation modification mediated by downstream O-methyltransferases (OMTs). A lower cellular pH provides a stable chemical environment for color manifestation. The shift from green to purple leaves stems from

an increase in total anthocyanin content and differential activation of downstream methylation and glycosylation modification pathways among different germplasms. Metabolomics analysis clarified the material basis and key metabolic features of the purple leaves, which are primarily governed by peonidin and malvidin derivatives. The greening to purple transition is attributed to decreased levels of chlorophylls and carotenoids, which reduces their masking effect on anthocyanin color, while the significant increase in anthocyanin content ultimately determines the purple leaf phenotype. The metabolomics analysis not only elucidated the chemical mechanism underlying purple leaf formation in this species but also identified germplasms like ZM2, which are rich in highly stable anthocyanins. This work lays the foundation for further transcriptomics studies to dissect the molecular regulatory network of the purple trait and provides metabolite markers and superior germplasm resources for color improvement and targeted breeding.

Supplementary Information

List of Abbreviations

Glossary of Terms

Abbreviation	Full Name
Cy	Cyanidin
Dp	Delphinidin
Pg	Pelargonidin
Pn	Peonidin
Pt	Petunidin
Mv	Malvidin
TA	Total Anthocyanins
Chl a	Chlorophyll a
Chl b	Chlorophyll b
Car	Carotenoid
OMT	O-Methyltransferase
F3'5'H	Flavonoid 3',5'-Hydroxylase
UFGT	UDP-glucose: flavonoid glucosyltransferase

Additional file: Table 1.Sample Information.**Table 2.**Leaf color phenotypic characteristics in *N. grossedentata*.**Fig. 12** Photosynthetic pigment contents in *N. grossedentata* with different leaf colors. **Fig. 13** Comparison of total anthocyanin content in *N. grossedentata* materials with different.**Fig. 3** pH in *N. grossedentata* materials with different leaf colors.**Fig. 4** Correlation of key indicators associated with leaf color formation in *N. grossedentata*.**Fig 5** Anthocyanin composition and content in different materials.**Fig. 6** Heatmap of Anthocyanin Composition Differences Among Five *N. grossedentata* Materials.**Fig. 7** Overlap of differential metabolites identified in pairwise comparisons between LM2 and the other four *N. grossedentata* samples with varying leaf colors.**Fig. 8** Key anthocyanin glycosides that were accumulated in the purple leaves of *N. grossedentata*.**Fig. 9** The expression distribution of differentially expressed metabolites across different comparison groups.**Fig. 10** KEGG enrichment analysis of differential metabolites among different leaf-color comparison groups in *N. grossedentata*.**Fig. 11** Heatmap of correlation matrix among leaf color parameters in *N. grossedentata*.

Declarations

Method statement

The investigation and collection of leaf samples of *N. grossedentata* this study have been approved by the local regulatory authorities. The leaves of *N. grossedentata* identified by Professor Weiqiang Qin of Jishou University as belonging to the genus *Nekemias* in the family Vitaceae. The voucher specimens have been deposited in the Herbarium of the College of Life Sciences, Jishou University, with voucher numbers JSU-NG15, JSU-NG27, JSU-NG65, JSU-NG79, and JSU-NG80.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The data used to support the findings of this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

Y.W., M.Y., L.T.; Conceptualization, Investigation, Formal analysis, C.Q. Methodology, Validation, C.Z.X.; Data curation, Visualization, J.T. Investigation, Resources, Y.W., Y.B.S.; Writing Original Draft, Supervision, C.L.T.; Funding acquisition, Writing Review & Editing. All authors have read and approved the final version of the manuscript.

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Not applicable.

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