



Flavonoid extracts from chrysanthemum with appropriate anthocyanins turn blue when exposed to iron ions

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ABSTRACT

Although some species that accumulate only cyanidin (Cy) in nature can produce blue flowers through iron ions, there has been no evidence of blue chrysanthemums being generated in this manner. This study revealed that flavonoid extracts from the ray florets of the chrysanthemum cultivar ‘Wandai Fengguang’ turned blue when exposed to Fe^{3+} . Samples that could turn blue were labeled as CB (Cy-determined blue flowers), while samples that did not turn blue were labeled as CN (Cy-determined non-blue flowers). After a series of experiments, a stable screening system was established using flavonoid extracts containing NaAc buffer at pH 5.5 and a total anthocyanin concentration (TAC) of $30 \mu\text{mol} \cdot \text{L}^{-1}$, and the addition of Fe^{3+} from 0 to $0.25 \mu\text{mol} \cdot \text{L}^{-1}$ allowed for the selection of five CB samples from 39 chrysanthemum cultivars. All five CB samples exhibited flower color phenotypes that belonged to Cluster-I with redness (a^*) values ranging from 29.03 to 45.99, yellowness (b^*) values from -11.31 to 3.77 , and brightness (L^*) values from 29.07 to 45.99. Additionally, the ratio of TAC to total luteolin concentration (TLC) was found to be a critical factor for distinguishing between CB and CN samples. To realize the desired blue hue in the flavonoid extracts with the participation of Fe^{3+} , a TAC to TLC ratio of 2.25 and above is required. Moreover, the protoplasts and ray florets of CB samples that turned blue with the involvement of Fe^{2+} showed great potential for cultivating blue chrysanthemums through ferric–anthocyanin chelate. Overall, this study reveals that blue flowers can be cultivated through the increase in the iron ion concentration, combined with the accumulation of Cy.

Keywords: *Chrysanthemum* × *morifolium*; Cyanidin; Copigment; Ferric-anthocyanin chelate; Blue flower

1. Introduction

Chrysanthemum (*Chrysanthemum* × *morifolium*) is a globally valued commodity flower crop that is known for its diverse varieties, resilience, and beauty. The rarity of blue flowers in nature has increased their popularity, as they are often associated with nobility

and mystery. Because of the absence of blue cultivars in natural chrysanthemum taxonomy (Sun et al., 2010; Hong et al., 2012; Dai and Hong, 2016; Lou et al., 2021; He et al., 2023), the creation of blue chrysanthemums remains an important breeding objective.

Previous studies focused on cultivating blue chrysanthemums through the delphinidin (Dp) pathway. Huang et al. (2013)

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attempted to reconstruct this pathway by overexpressing the flavonoid 3',5'-hydroxylase (F3'5'H) gene from *Senecio cruentus* and downregulating the flavonoid 3'-hydroxylase (F3'H) gene to prevent cyanidin (Cy) synthesis. In spite of the attempts undertaken, the transgenic chrysanthemums did not manifest blue petals owing to the elevated levels of Cy. In order to create blue/violet chrysanthemum flowers, researchers genetically engineered Dp production by transforming the plants with a chimeric pansy F3'5'H gene under the control of floral-specific promoters (Brugliera et al., 2013). Although the petal-specific flavanone 3-hydroxylase (F3H) promoter in chrysanthemums was controlled by the 5'-untranslated region of the alcohol dehydrogenase gene as a translational enhancer, and the significant accumulation of Dp was achieved through the simple overexpression of the campanula F3'5'H, the petal color remained insufficiently blue (Noda et al., 2013). Noda et al. (2017) successfully created true blue-colored chrysanthemum flowers by introducing the butterfly pea uridine diphosphate (UDP)-glucose: anthocyanin 3',5'-O-glucosyltransferase gene. However, there was still a significant difference between the flower color and the desired shade of sapphire blue. In addition, when co-expressing CtA3'5'GT and OhF3'5'H in the CmF3'H mutant, the transgenic chrysanthemum flowers remained purple (Han et al., 2021), which indicates that the degree of blue shift in flower color is greatly influenced by the source of F3'5'H. Thus, further research is needed.

In addition to Dp and high pH, metal complexation is another method for achieving blue flowers (Yoshida et al., 2009). The examination of metal complexes in various blue flowers has revealed two modes of action for metal ions. The first involves the formation of stoichiometric metal–pigment–copigment complexes in which each component is present in a specific stoichiometric ratio. It has been reported that comelinin, protocyanin, protodelphin, cyanosalvianin, and nemophilin form such complexes (Kondo et al., 2001; Shoji et al., 2006; Takeda, 2006; Mori et al., 2008; Shiono et al., 2008; Yoshida et al., 2015). For example, sapphire blue protocyanin in *Centaurea cyanus* comprises six molecules of Cy, six molecules of apigenin, one molecule of Fe^{3+} , one molecule of Mg^{2+} , and two molecules of Ca^{2+} (Takeda et al., 2005; Takeda, 2006). The second mode of action comprises non-stoichiometric metal–pigment–copigment complexes that influence flower color (Shoji et al., 2006; Yoshida et al., 2006, 2021). Fe^{3+} and kaempferol are crucial in the formation of Cy-based blue pigments in *Meconopsis grandis* and *Corydalis ambigua* (Yoshida et al., 2006, 2021). The major metal ions involved in complex formation in blue flowers are Mg^{2+} , Al^{3+} , and Fe^{3+} . When Cy is the primary anthocyanin, Fe^{3+} is necessary to produce blue flowers, whereas Mg^{2+} can turn flowers blue when Dp is the primary anthocyanin (Yoshida et al., 2009). It can be inferred that Fe^{3+} plays a crucial role in the accumulation of Cy and in the generation of blue flowers.

However, to the best of our knowledge, there are few reports of the Cy pathway being used in the cultivation of blue flowers until now. The authors previously conducted initial trials that revealed that flavonoid extracts derived from the ray florets of the chrysanthemum cultivar 'Wandai Fengguang' changed color from reddish-purple to blue when exposed to Fe^{3+} .

Samples that exhibited this property were referred to as CB (Cy-determined blue flowers), while those that did not were labeled as CN (Cy-determined non-blue flowers). However, a reliable screening system had yet to be established. As a result, we selected 39 cultivars of the pink-purple-family chrysanthemums, in which Cys are the primary color-presenting substances, to investigate the color change of Cys in chrysanthemums under the action of Fe^{3+} and to screen CB samples from them. The investigation focused on analyzing the influence of several factors, including the pH level of the buffer solution, the total anthocyanin concentration (TAC) in the flavonoid extracts, and the concentration of Fe^{3+} . The objective was to establish a screening system for chrysanthemum samples. The study also emphasized the significance of the ratio of the TAC to the total luteolin concentration (TLC) in determining whether the chrysanthemum flavonoid extract solution could turn blue. Additionally, it was demonstrated that the vacuoles and ray florets of CB samples could indeed turn blue upon the addition of iron ions.

This study is a preliminary attempt to cultivate blue chrysanthemums using the Cy pathway, making blue flower cultivation no longer restricted to the Dp pathway. The Cy pathway provides a new approach for improving the color of flowers that accumulate Cy but cannot produce blue flowers. This work created a stable CB sample screening system that enabled fast and effective screening, saving time for CB sample screening in more varieties. Furthermore, it was found that after the application of iron, the vacuoles and petals of CB samples showed localized blueness in potted chrysanthemum ray florets due to iron supplementation, which is of great significance for the breeding of horticultural plants with blue flowers.

2. Materials and methods

2.1. Materials

All plant materials were acquired from the Beijing Greenland Maintenance and Management Service Center (Beijing, China). Cyanidin-3-O-glucoside chloride (B21171), luteolin (B20888), apigenin (B20981), cyanidin-3-O-(6''-malonylglucoside) chloride (3-caffeoylquinic acid) (B22264), and chlorogenic acid (B20782) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Methanol, formic acid, acetonitrile, trifluoroacetic acid, phosphate buffer, sodium acetate, glacial acetic acid, dimethyl sulfoxide (DMSO), cellulase R-10, macer-ozyme R-10, fluorescein diacetate (FDA), mannitol, bovine serum albumin (BSA), 2-morpholinoethanesulphonic acid (MES), FeCl_3 , NaCl, CaCl_2 , KCl, and ferrous ammonium sulfate were obtained from Beijing BioDee Biotechnology Co., Ltd. (Beijing, China).

2.2. Methods

2.2.1. Determination of flower color phenotypes

Fresh ray florets of the chrysanthemum cultivar 'Wandai Fengguang' were collected at the blooming stage and placed in a well-lit area, avoiding direct exposure to sunlight. The color of the flowers was matched to the center of the ray florets using the

Royal Horticultural Society Color Chart (RHSCC), and the process was repeated three times. Furthermore, a spectrophotometer NF333 (Nippon Denshoku Industries, Tokyo, Japan) was used to determine the color phenotype of the flowers. Three sets of samples were taken for each variety of capitulum, and three to five ray florets were stacked and laid flat on white paper for each set. The light-collecting hole of the colorimeter was aligned with the center of the petal surface, and the brightness (L^*), redness (a^*), and yellowness (b^*) values of all samples were measured. Three capitula were determined for each cultivar, and at least three sets of data were generated from each capitulum (Hong et al., 2012).

2.2.2. Determination of TAC

Ray florets (0.1 g) were crushed into a fine powder in a mortar and pestle with liquid nitrogen. A suitable quantity of extraction solution (methanol: water: formic acid: trifluoroacetic acid = 70: 27: 2: 1, V/V) was added immediately. The extracts were rapidly shaken and mixed at 4 °C for 24 h. During this period, the extraction solution was added until the precipitation became colorless. All extracts were pooled and filtered through a 0.45- μm filter into centrifuge tubes (Sun et al., 2010). The absorbance values of each cultivar at 525 nm were recorded using a Biomate 3S UV–Visible Spectrophotometer (Thermo Scientific, Waltham, MA, USA) scanned at 300–600 nm with a scanning interval of 1 nm and a quartz cup optical diameter of 1 cm.

The cyanidin-3-O-glucoside chloride standard was accurately weighed, dissolved in extraction solution (Sun et al., 2010), and then diluted to a concentration range of 31.25–500.00 $\mu\text{g}\cdot\text{mL}^{-1}$ to construct calibration curves at 525 nm. The regression equation was $Y = 2.8535X + 0.0263$ ($R^2 = 0.9915$). The TAC in $\text{mg}\cdot\text{g}^{-1}$ FW (fresh weight) per gram of fresh petals was calculated separately using a semiquantitative method (Deng, 2020).

2.2.3. Determination of total carotenoid concentration

The total carotenoids were extracted and determined following the procedures used in previous research with some modifications (Burgos et al., 2009). Briefly, approximately 100 mg of freeze-dried ray floret samples were added to 2 mL of extraction solution (acetone: petroleum ether = 1: 4, V/V) for 24 h in the dark at 4 °C. Next, the samples were centrifuged at 13 000 $\text{r}\cdot\text{min}^{-1}$ for 1 min; then the absorbance of the supernatant was measured at 450 nm using the Biomate 3S UV–Visible Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The extinction coefficient for mixtures of carotenoids is 2 500.

2.2.4. Preparation of flavonoid extract solutions

Ray florets (5–10 g) were crushed into a fine powder in a mortar and pestle with liquid nitrogen. The resulting powder was quickly added to 100 mL of an extraction solution for extraction (Sun et al., 2010). The extracts were then centrifuged at 12 000 $\text{r}\cdot\text{min}^{-1}$ for 10 min, and the supernatant was filtered through a Whatman filter paper. The obtained extracts were concentrated using a rotary evaporator apparatus (Lab Tech EV311, Beijing LabTech Instruments Co., Ltd., Beijing, China) at 58 °C. The samples were then frozen in liquid nitrogen and freeze-dried in a freeze dryer (LGJ-10, Beijing Songyuan Huaxing Technology Development Co., Ltd., Beijing, China) at –50 °C for 72 h until a constant weight was reached. The flavonoid extracts

were obtained by dissolving freeze-dried extracts in a 0.1 $\text{mol}\cdot\text{L}^{-1}$ phosphate buffer solution, and the determination of TAC was performed using the pH-differential method (Giusti and Wrolstad, 2001).

2.2.5. Establishment of a CB sample screening system and CB sample screening

(1) Determination of the optimum pH of the buffer

The reaction was conducted in a 0.2 $\text{mol}\cdot\text{L}^{-1}$ NaAc buffer. The same volume of flavonoid extracts from the chrysanthemum cultivar ‘Wandai Fengguang’ was added to NaAc buffers containing Fe^{3+} at different pH values (5.0, 5.5, 6.0, 6.5, and 7.0). Digital images were obtained under fixed positions under the same conditions (light source, room, and camera). The color data of L^* , a^* , and b^* at the measured sites were collected using Adobe Photoshop CC 2019 (Adobe Systems Inc., San Jose, CA, USA) as the mean value of the three images and processed using ColorMate software 5 (Nippon Denshoku Industries, Tokyo, Japan). The absorbance values of the solutions were also measured using an ultraviolet (UV) spectrophotometer between wavelengths of 400–700 nm.

(2) Determination of the molar concentration ratio of TAC to Fe^{3+}

The reaction was conducted in a 0.2 $\text{mol}\cdot\text{L}^{-1}$ NaAc buffer at pH 5.5. Flavonoid extracts with different TAC (10, 20, 30, and 40 $\mu\text{mol}\cdot\text{L}^{-1}$) from the chrysanthemum cultivar ‘Wandai Fengguang’ were mixed with different concentrations of FeCl_3 (0, 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1 $\mu\text{mol}\cdot\text{L}^{-1}$). Image acquisition, color analysis, and spectral analysis were performed as previously described.

(3) Selection of CB samples

Flavonoid extracts with TAC of 30 $\mu\text{mol}\cdot\text{L}^{-1}$ from the remaining 38 chrysanthemum cultivars were mixed with different concentrations of FeCl_3 (0, 0.03125, 0.0625, 0.125, 0.25 $\mu\text{mol}\cdot\text{L}^{-1}$) to screen the CB samples. Image acquisition and color analysis were completed as previously described.

2.2.6. Analysis of flavonoid fractions between CB and CN samples

The extraction of flavonoids was performed as described in Section 2.2.4. Flavonoids were separated using a 2.1 mm $\times$ 100 mm Waters® ACQUITY™ 1.7 μm HSS C18 column (Waters Corporation, Milford, MA, USA) on a Waters ACQUITY Ultra Performance Liquid Chromatography system (UPLC I-CLASS, Waters). The column temperature was set at 35 °C, and photodiode array spectra were recorded from 100 to 1 200 nm. The analysis was realized with gradient elution using water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) as the mobile phase at a flow rate of 0.4 $\text{mL}\cdot\text{min}^{-1}$. The gradient conditions for the mobile phase were as follows: 95% A at 0 min, 85% A at 2 min, 80% A at 7 min, 70% A at 10 min, 10% A at 11 min, 95% A at 11.1 min, and 95% A at 13 min.

The qualitative analysis of flavonoids was performed using a Xevo™ TQ-MS triple quadrupole mass spectrometer (Waters) connected to an ACQUITY UPLC instrument (UPLC I-CLASS, Waters). Ionization was achieved using an electrospray ion (ESI) source in both positive ion (PI) and negative ion (NI) modes. The ionization source parameters were set as follows: capillary voltage, 4.00 (+)/4.00 (–); cone voltage, 30 V; desolvation gas flow, 650 L·h^{–1}; cone gas flow, 50 L·h^{–1}; collision gas flow, 0.15 (+)/0.15 (–) mL·min^{–1}; collision energy, 15 (+)/15 (–) eV; desolvation temperature = 350 °C; and scan range 100 to 1 200 (*m/z*) units. The MassLynx V 4.1 analytical software (Waters) was used for system control and data processing.

The concentration of flavonoids in acetonitrile extracts was quantified by UPLC analysis with a detection wavelength of 525 nm for anthocyanins and 350 nm for other flavonoids, based on the calibration curves obtained from the dilution series of cyanidin-3-O-(6''-malonylglucoside) chloride and luteolin, respectively. The regression equations were $Y = 1\,399\,410X - 1\,955.3$ ($R^2 = 0.9991$) and $Y = 1\,254\,670.86X - 41.64$ ($R^2 = 1.00$) for cyanidin-3-O-(6''-malonylglucoside) chloride and luteolin, respectively, showing good linearity between the concentration and the peak areas. The results were provided in milligrams of cyanidin-3-O-(6''-malonylglucoside) equivalents for anthocyanins and luteolin equivalents for other flavonoids per gram of fresh weight (mg·g^{–1} FW) (Deng, 2020).

2.2.7. Reconstruction of the blue pigment of *chrysanthemum* in vitro

Standard solutions of each analyte, including cyanidin-3-O-(6''-malonylglucoside) chloride, luteolin, apigenin, and chlorogenic acid, were dissolved in DMSO at a concentration of 1 mg·mL^{–1} in order to prepare the reaction. The reaction was carried out in a 0.2 mol·L^{–1} NaAc buffer at pH 5.5. To begin, cyanidin-3-O-(6''-malonylglucoside) chloride at a concentration of 0.2 mg·mL^{–1} was mixed with varying concentrations of Fe³⁺ (0, 0.03125, 0.0625, 0.125, 0.25, and 0.5 μmol·L^{–1}). Afterward, different concentrations of luteolin, apigenin, and chlorogenic acid (0.025, 0.05, 0.1, and 0.2 mg·mL^{–1}) were added to the solution containing mixed cyanidin-3-O-(6''-malonylglucoside) chloride (0.2 mg·mL^{–1}) and Fe³⁺ (0.125 μmol·L^{–1}). The acquired images were analyzed to determine their color and spectral properties using previously established techniques.

2.2.8. Production of blue color in vivo

(1) Production of blue color in protoplasts

Protoplast preparation: All glassware, plastic tubes, tips, pipettes, and other materials used for protoplast preparation were autoclaved and sterilized. All reagents were prepared using double-distilled water. The petals of the *chrysanthemum* cultivars 'Wandai Fengguang', 'Ziyan Fanfei', 'Dongli Zijiao', 'Dongli Fenzhuang', and 'Dongli Qiushi' were sterilized for 30 s using 75% ethanol and then rinsed two to three times with double-distilled water. The fresh flowers were cut into strips with a width of about 1 mm, and the strips were digested in an enzymatic digestion solution (4% cellulase, 0.4% macerozyme, 0.6 mol·L^{–1} mannitol, 20 mmol·L^{–1} KCl, 20 mmol·L^{–1} MES, 10 mmol·L^{–1} CaCl₂, and 5 mg·mL^{–1} BSA) at 28 °C with shaking at 55 r·min^{–1} for 8 h. The

reaction was terminated by doubling the volume of W5 buffer (4 mmol·L^{–1} MES, 154 mmol·L^{–1} NaCl, 125 mmol·L^{–1} CaCl₂, 5 mmol·L^{–1} KCl, pH 5.7), and the protoplasts were filtered using a sieve with a pore size of 70 μm and centrifuged (300×*g*, 5 min). The precipitate was washed two more times and then resuspended in the W5 buffer.

Coloring of protoplasts with iron: ferrous ammonium sulfate was prepared in solutions with concentrations of 0.001, 0.01, and 0.1 mmol·L^{–1}. The same volume of Fe²⁺ at different concentrations was added to the corresponding W5 buffer with an equal amount of suspended protoplasts.

It was necessary to first extract 100 μL of protoplasts with appropriate density into a small centrifuge tube to detect the vitality of protoplasts. Next, 2.4 μL of FDA was added, which can be prepared by dissolving 5 mg of FDA in 1 mL of acetone and storing it in the dark at 4 °C. The mixture was allowed to stand for 5 min at 28 °C in darkness. The protoplasts were transferred onto a glass slide and examined under an inverted fluorescence microscope (Olympus IX71, Olympus, Tokyo, Japan). The *L**, *a**, and *b** values of the protoplast colors were collected using the Photoshop color pickup function. The color definition was consistent with the previous method.

(2) Production of blue color in petals

Treatment of ray florets with ferrous ions: For the control group, the bases of full-flowered 'Wandai Fengguang' ray florets were placed in a sterilized solid medium containing 1% sucrose and 7 g·L^{–1} of agar at a pH of 5.9. The same procedure was followed for the experimental group, but the medium also contained 50 μmol·L^{–1} ferrous ammonium sulfate. The ray florets were recorded after 0 h and 48 h of placing the ligule flower on the medium, and epithelial photographs were captured using a SDPTOP CX40 microscope (Sunny Optical Technology (group) Co., Ltd., Zhejiang, China).

Ferrous ion spray for capitula of potted flowers: During the incomplete opening of 'Wandai Fengguang' (ray florets were protruding from the involucre but had not yet unfolded), 0 or 50 μmol·L^{–1} ferrous ammonium sulfate was sprayed and the growth of the flower petals was observed after 7 d. Epithelial photographs were taken using a SDPTOP CX40 microscope (Sunny Optical Technology).

2.2.9. Data analysis

The phenotypic values of ligulate floret color were measured using a colorimeter and systematically clustered using IBM SPSS Statistics 25 software (IBM Corporation, Armonk, NY, USA). The furthest-neighbor method was selected for the cluster analysis. The remaining data were analyzed and plotted using Origin 2019 (Origin Lab Corp., Northampton, MA, USA).

3. Results

3.1. Analysis of flower color and pigment in *chrysanthemums*

This investigation aimed to establish the correlation between flower color phenotype and pigment concentration in 39 *chrysanthemum* cultivars. The results of colorimeter measurements using the Munsell color system indicated that only one cultivar

belonged to the red group, whereas the remaining 38 cultivars were categorized as red-violet (Table S1). However, the spatial arrangement of the different cultivars based on CIELAB color coordinates was distinct (Fig. S1). In conclusion, the 39 chrysanthemum cultivars were classified into three groups via hierarchical clustering analysis of the L^* , a^* , and b^* values (Fig. 1, A).

The flower color phenotype of the three groups exhibited important differences. Cluster-I showed a ray floret with low brightness, high redness, and low yellowness, as evidenced by the range of L^* values from 29.07 to 45.99, a^* values from 29.03 to 45.99, and b^* values from -11.31 to 3.77 . Cluster-II showed a ray floret with moderate brightness, redness, and yellowness, as shown by the range of L^* values from 41.82 to 57.54, a^* values from 24.06 to 35.75, and b^* values from -9.19 to 3.77 . Cluster-III showed a ray floret with high brightness, low redness, and high yellowness, as evidenced by the range of L^* values from 61.98 to 77.10, a^* values from 3.41 to 22.12, and b^* values from -5.7 to 2.71 (Table S1 and Fig. 1, B).

The total carotenoid concentration was similar across the three clusters; however, there was considerable variation within each cluster, with carotenoid concentration being low in all clusters (Fig. 1, C and Fig. S2). Therefore, the influence of carotenoids on flower color in the 39 chrysanthemum cultivars was not examined in subsequent experiments. The total anthocyanin concentration was significantly higher in Cluster-I than in Cluster-II and Cluster-III (Fig. 1, D).

3.2. Determination and selection of CB sample screening conditions

The pH, TAC, and Fe^{3+} concentration must be considered to screen for potential CB samples. However, if all chrysanthemum cultivars were used as materials for direct screening, the process would be both complicated and time-consuming. After several experiments, it was found that the flavonoid extracts of 'Wandai Fengguang' could turn blue in the presence of Fe^{3+} , and was therefore considered a valid CB sample. To efficiently establish a stable screening system and obtain more CB samples, 'Wandai Fengguang' was used as a material to establish a CB sample screening system.

3.2.1. Optimal pH value of the buffer solution

Further experiments were conducted to evaluate how pH affects the color of flavonoid extracts after the addition of Fe^{3+} and to determine the optimal pH for CB sample screening. The investigation revealed that the color of the solution remained unaffected by pH changes, regardless of Fe^{3+} addition (Fig. 2, A). Nevertheless, the CIELAB color data and spectral data detected variations that escaped the naked eye (Fig. 2, B, C and Table S2).

When iron was not added, there were increases in L^* and b^* and decreases in a^* as the pH was raised from 5 to 7. Following the addition of iron, there was a tendency for L^* to first decrease and then increase, while b^* gradually increased and a^* gradually decreased as the pH rose from 5 to 7 (Table S2). The absorption spectra demonstrated that in the absence of iron, the maximum absorption peak underwent varying degrees of rightward shift as the pH increased, accompanied by a rise in absorbance.

Specifically, raising the pH from 5 to 5.5 and subsequently to 6 produced little change in the maximum absorption peak but led to a gradual boost in absorbance. In contrast, raising the pH further to 6.5 and then 7 resulted in an increase in the maximum absorption peak, while the absorbance tended to initially decline and subsequently increase. The absorbance value showed a trend of decreasing and then increasing, while after the iron addition, the maximum absorption peak showed a trend of increasing and then decreasing. It reached its highest value at pH 6. When the pH increased from 5 to 6, the absorbance value increased gradually, hitting the maximum at pH 6. When the pH increased from 6 to 6.5 and then to 7, the absorbance values initially decreased and then increased (Fig. 2, B). The degree of red shift and the color enhancement effect were calculated both before and after iron addition to determine the influence of the pH level on the color enhancement effect and the degree of red shift following iron addition. The results indicated that the degree of red shift was the most prominent at pH values of 5.5 and 6.5, while the color enhancement effect was the strongest at pH values of 5.5 and 6.0 (Fig. 2, C). Subsequently, the experiments were conducted in a buffer with a pH value of 5.5.

3.2.2. Ratio of TAC to Fe^{3+}

In order to investigate the effect of the anthocyanin to Fe^{3+} ratio on color rendering, this work compared the color alteration of flavonoid extracts blended with Fe^{3+} at several molar concentrations in conjunction with different TAC. The findings revealed that the color of flavonoid extracts gradually transitioned toward blue as the Fe^{3+} concentration increased while maintaining a constant TAC. For example, at a TAC of $30 \mu\text{mol} \cdot \text{L}^{-1}$, the solution changed color from purple-red to blue-purple upon adding Fe^{3+} at a concentration of $0.03125 \mu\text{mol} \cdot \text{L}^{-1}$, followed by blue at a higher Fe^{3+} concentration of $0.0625 \mu\text{mol} \cdot \text{L}^{-1}$, and ultimately blue-green upon further increasing the Fe^{3+} concentration to 0.125 or $1 \mu\text{mol} \cdot \text{L}^{-1}$ (Fig. 2, D and Table S3).

The concentration of Fe^{3+} had a direct influence on the absorbance of the maximum absorption peak of anthocyanin. The absorbance gradually increased from a range of -0.02 – 0.05 to 0.53 – 1.69 , indicating a hyperchromic effect and a red-shift phenomenon (Table S4). It is worth noting that a high TAC is required for the solution to exhibit a blue color, along with a specific molar ratio of TAC to Fe^{3+} . For example, at a molar ratio of 320 of TAC to Fe^{3+} , a TAC solution of 10 or $20 \mu\text{mol} \cdot \text{L}^{-1}$ did not exhibit blue coloration. Only when the TAC reached $40 \mu\text{mol} \cdot \text{L}^{-1}$ did the solution exhibit the blue hue (Fig. 2, D). During this study, colorations of blue-green, green, and yellow-green were observed, indicating that anthocyanin is not the only chemical responsible for color presentation. Spectral analysis revealed that in addition to anthocyanins, flavonoids played a role in the color alteration of flavonoid extracts following Fe^{3+} addition. This was confirmed by a rightward shift in the λ_{max} and an increase in absorbance values (Fig. S3).

To sum up, the study findings suggest that flavonoid-based solutions exhibit color variations in response to different concentrations of Fe^{3+} , shifting from purple-red to blue-green. The coloration of flavonoid extracts stabilized at $0.25 \mu\text{mol} \cdot \text{L}^{-1}$, prompting the use of Fe^{3+} concentrations within the range of 0 to

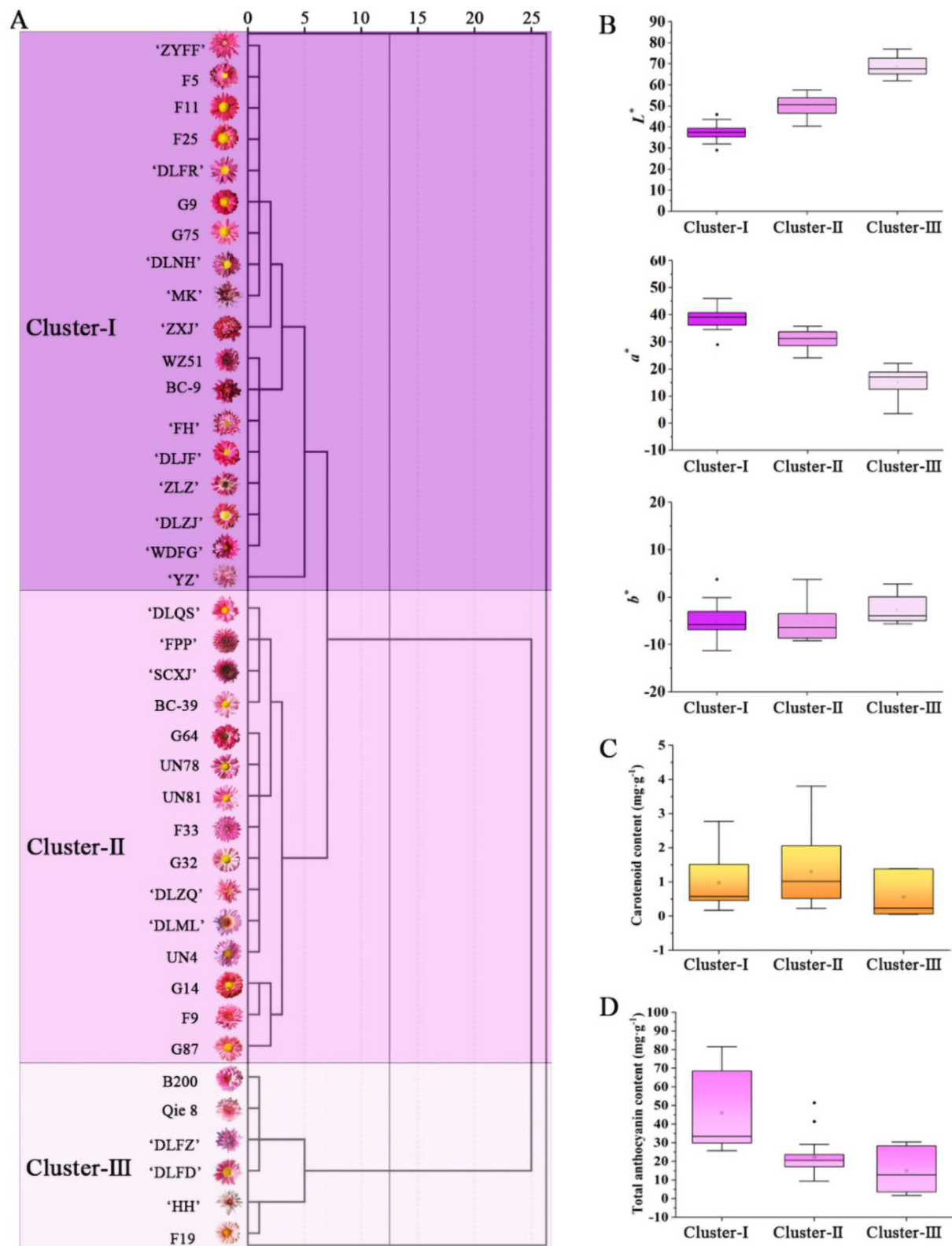


Fig. 1 Hierarchical clustering analysis of flower color and pigmentation analysis of 39 chrysanthemum cultivars

A: Hierarchical clustering analysis was conducted on the floral color phenotypes of 39 chrysanthemum cultivars; B: A comparative analysis was performed on the L^* , a^* , b^* values of three groups of chrysanthemums; C: The total carotenoids in three groups; D: The total anthocyanins in three groups. Explanation of abbreviated cultivar names in the figure: 'HH': 'Hehua'; 'DLFZ': 'Dongli Fenzhuang'; 'DLFD': 'Dongli Fendai'; 'SCXJ': 'Sanceng Xiaojv'; 'DLQS': 'Dongli Qushi'; 'FPP': 'Fenpingpang'; 'ZXJ': 'Zixiangjv'; 'ZLZ': 'Zilouzi'; 'MK': 'Mokui'; 'DLZJ': 'Dongli Zijiao'; 'DLZQ': 'Dongli Zhiqiu'; 'FH': 'Fenhe'; 'DLML': 'Dongli Milian'; 'DLNH': 'Dongli Ninghong'; 'WDFG': 'Wandai Fengguang'; 'ZYFF': 'Ziyan Fanfei'; 'DLJF': 'Dongli Jiaofen'; 'DLFR': 'Dongli Furong'; 'YZ': 'Yanzhi'.

0.25 $\mu\text{mol}\cdot\text{L}^{-1}$ to screen CB samples based on the color of flavonoid extracts. Fig. 2 shows that flavonoid extracts exhibit various hues such as purple-red, blue-violet, blue, and blue-green at different Fe^{3+} concentrations with a TAC of 30 $\mu\text{mol}\cdot\text{L}^{-1}$, meeting the criteria for screening CB samples based on the flavonoid extract color. Consequently, flavonoid extracts possessing a TAC of 30 $\mu\text{mol}\cdot\text{L}^{-1}$ were chosen to further screen CB samples.

3.2.3. Selection of CB samples

The color of flavonoid extracts was evaluated after the addition of Fe^{3+} based on the screening criteria to ascertain the 38 remaining chrysanthemum cultivars. The results indicated that only four cultivars were CB samples, while the others were classified as CN samples (Fig. 3 and Table S5). Moreover, the flower color phenotype of all five CB samples belonged to Cluster-I (Fig. 3), indicating that they had high anthocyanidin concentration.

3.3. Analysis of flavonoid fractions in CB and CN samples

A total of five CB and eight CN samples were selected for qualitative and quantitative assessment to comparatively analyze the flavonoid fractions presented in CB and CN samples. The measurements were conducted at two different wavelengths, with five peaks recorded at 530 nm, all of which were identified as Cys. At 350 nm, 33 peaks were recorded, with five peaks being identified as luteolin, eight peaks as apigenin, five peaks as diosmetin, seven peaks as acacetin, one peak as quercetin, and another seven peaks as hydroxycinnamic acid (Table S6) (Lin and Harnly, 2010; Sun, 2010; Noda, 2018; Lu et al., 2020).

The results showed that A3 [cyanidin-3-O-(6''-O-malonyl) glucoside] and A4 [cyanidin 3-O-(3'', 6''-O-dimalonyl-glucopyranoside)] were the most abundant anthocyanins in both the CB and CN samples. The main flavonoids in the CB samples were F6 (luteolin-7-O-6''-malonylglucoside) or F12 (apigenin-7-O-6''-

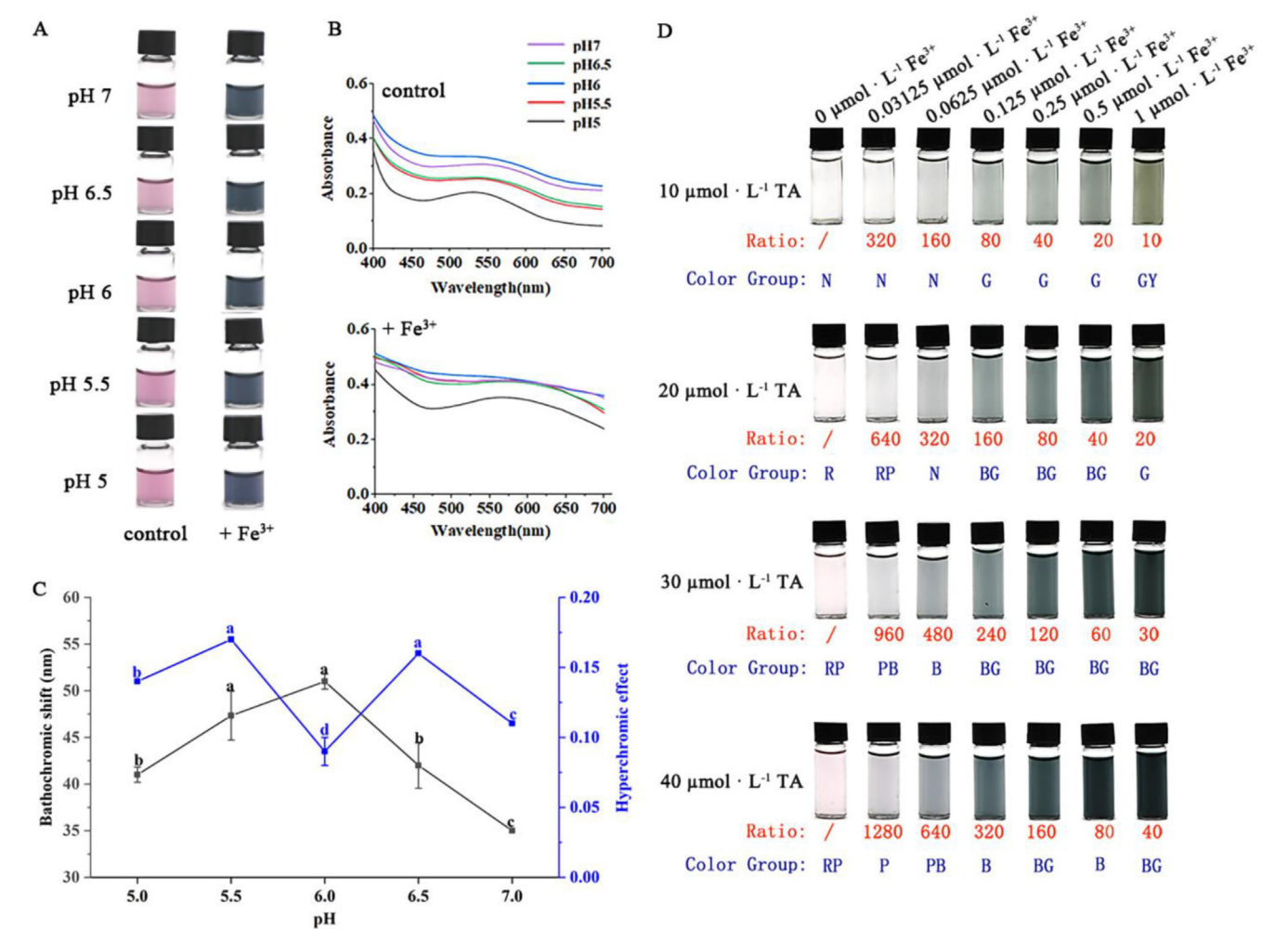


Fig. 2 Establishment of a CB sample screening system

(A–B): Changes of color (A) and spectrum (B) of flavonoid extract solutions of the chrysanthemum cultivar ‘Wandai Fengguang’ in different pH buffers and after the addition of Fe^{3+} ; C: Changes in the highest absorption peak and absorbance values under different pH conditions after the addition of Fe^{3+} ; D: The alterations in the color of flavonoid extract solutions are contingent upon the varying ratios of TAC and Fe^{3+} . Red characters indicate the ratio of TAC to Fe^{3+} ; blue characters indicate the color of taxa: N: No match; RP: Red Purple; R: Red; PB: Purple Blue; B: Blue; BG: Blue Green; G: Green; GY: Greenish Yellow. Different lowercase letters indicate significant differences at $P < 0.05$ (Duncan’s new multiple range test).

malonylglucoside isomer), the main flavonoids in most CN samples were F6 and F12, and the concentration of F2 (luteolin-7-O-glucoside) in the CN samples 'YZ' and WZ51 was also relatively high (Fig. 4, A, B).

The analysis of flavonoid types in the CB and CN samples revealed no significant difference. Although apigenin and caffeoylquinic acid are copigments present in high concentrations in chrysanthemums, there was no clear pattern between CB and CN samples. However, the ratio of TAC to TLC was observed to vary across samples. In CB samples, the values ranged from 2.25 to 6.87, while in CN samples they ranged from 0.47 to 1.62 (Fig. 4, C).

3.4. Copigment types and their concentrations determine the color of flavonoid extracts with Fe³⁺ involved in chrysanthemums

To confirm whether the varying ratio of TAC to TLC is a significant factor in the observed differences in the color of flavonoid extract solutions upon the introduction of Fe³⁺, and to explore the influence of three copigments (luteolin, apigenin, and

caffeoylquinic acid) on the blueness of Cy in the chrysanthemums, *in-vitro* experiments were conducted using standard substances, including cyanidin-3-O-(6''-malonylglucoside) chloride, luteolin, apigenin, and 3-caffeoylquinic acid.

The results revealed that the color of cyanidin-3-O-(6''-O-malonyl) glucoside was significantly influenced by the type of copigments. When only cyanidin-3-O-(6''-O-malonyl) glucoside was present, an increase in the Fe³⁺ concentration produced a bathochromic shift and a gradual increase in the hyperchromic effect, resulting in a blue-purple color (Fig. 5, A, B). However, when three copigments, namely luteolin, apigenin, and 3-caffeoylquinic acid, were added, all three caused a blue shift in color, while only luteolin and apigenin were capable of producing a blue appearance (Fig. 5, C, E, G). Furthermore, the UV spectrum indicated that the effect of the three copigments on cyanidin-3-O-(6''-O-malonyl) glucoside was ranked in the following order from strongest to weakest: luteolin, followed by apigenin, and 3-caffeoylquinic acid (Fig. 5, D, F, H).

The copigment concentration had a significant influence on the color of cyanidin-3-O-(6''-O-malonyl) glucoside. Specifically, when



Fig. 3 Screening results of sample CB samples

Blue characters indicate the color of taxa: RP: Red Purple; R: Red; PB: Purple Blue; B: Blue; BG: Blue Green; G: Green; GY: Greenish Yellow; Y: Yellow; YR: Yellowish Red. From left to right, the imposed concentrations of Fe³⁺ are 0, 0.03125, 0.0625, 0.125, and 0.25 μmol·L⁻¹. The explanation of abbreviated cultivars names in the figure is the same as above. The meaning of Cluster-I, Cluster-II, and Cluster-III in the Fig. 3 is the same as that of the annotations in Fig. 1.

the ratio of cyanidin-3-O-(6''-O-malonyl) glucoside to luteolin was 8:1, the resulting color was blue in the presence of Fe³⁺ (Fig. 5, C). However, as the concentration of luteolin increased, the color shifted from blue to yellow-green and then to yellow (Fig. 5, C). Similarly, when the ratio of cyanidin-3-O-(6''-O-malonyl) glucoside to apigenin was 4:1, the color was blue in the presence of Fe³⁺ (Fig. 5, E). When the concentration of apigenin was increased to

match that of cyanidin-3-O-(6''-O-malonyl) glucoside, the color of the latter changed to reddish yellow (Fig. 5, E).

In summary, luteolin had the most significant effect on the coloring of cyanidin-3-O-(6''-O-malonyl) glucoside when Fe³⁺ was present, compared to apigenin and 3-caffeoylquinic acid. It is imperative to maintain an appropriate ratio of TAC to TLC for the flavonoid extracts to show a blue hue after Fe³⁺ addition.

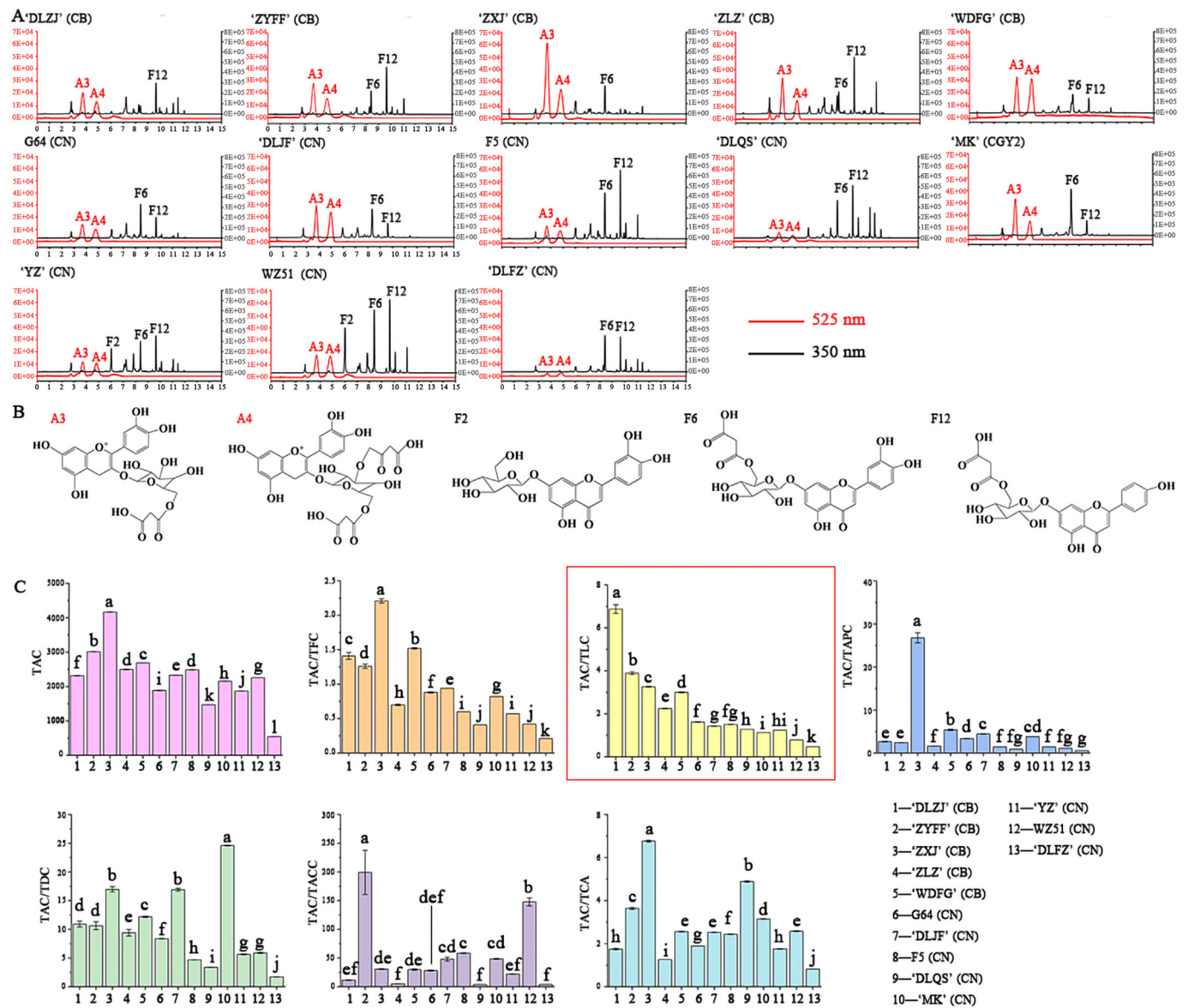


Fig. 4 Flavonoid fractions in CB and CN samples

A: The chromatogram of anthocyanins and flavonoids of different samples; B: Structural diagram of main anthocyanidin and flavonoids; C: Total anthocyanidin concentration in different samples and the ratio of total anthocyanidin to flavonoids and other components; TAC: total anthocyanin concentration; TFC: total flavonoid concentration; TLC: total luteolin concentration; TAPC: total apigenin concentration; TDC: total diosgenin concentration; TACC: total acacetin concentration; TCA: total caffeoylquinic acid concentration. Different lowercase letters indicate significant differences at $P < 0.05$ (Duncan's new multiple range test). The explanation of abbreviated cultivars names in the figure is the same as above.

3.5. CB samples gain blue coloration with iron ions in vivo

Because it is difficult for plants to absorb and utilize Fe^{3+} , Fe^{3+} could not be directly applied to plants. Fe^{2+} is easily absorbed by plants and can be oxidized to Fe^{3+} , which then reacts with anthocyanins (Fig. S4). Therefore, Fe^{2+} was chosen to study the effect of iron ions on CB samples in vivo.

3.5.1. Protoplasts appear blue in the presence of iron ions

The incorporation of Fe^{2+} into the petal protoplasts of the CB samples ‘Wandai Fengguang’, ‘Ziyan Fanfei’, and ‘Dongli Zijiao’

as well as the CN samples ‘Dongli Fenzhuang’ and ‘Dongli Qiushi’ resulted in the observation of a significant number of blue cells in the CB samples and blue-green cells in the CN samples (Fig. 6, A and Fig. S5). In a low-concentration Fe^{2+} environment, lighter cells showed a blue shift in color. When the Fe^{2+} concentration was increased, darker cells showed a blue shift, and the b^* of protoplasts gradually decreased with the increase of Fe^{2+} concentration (Fig. 6, B), which was consistent with the results of in-vitro pigment reconstruction. As the Fe^{2+} concentration increased, the number of protoplasts decreased, and the blue

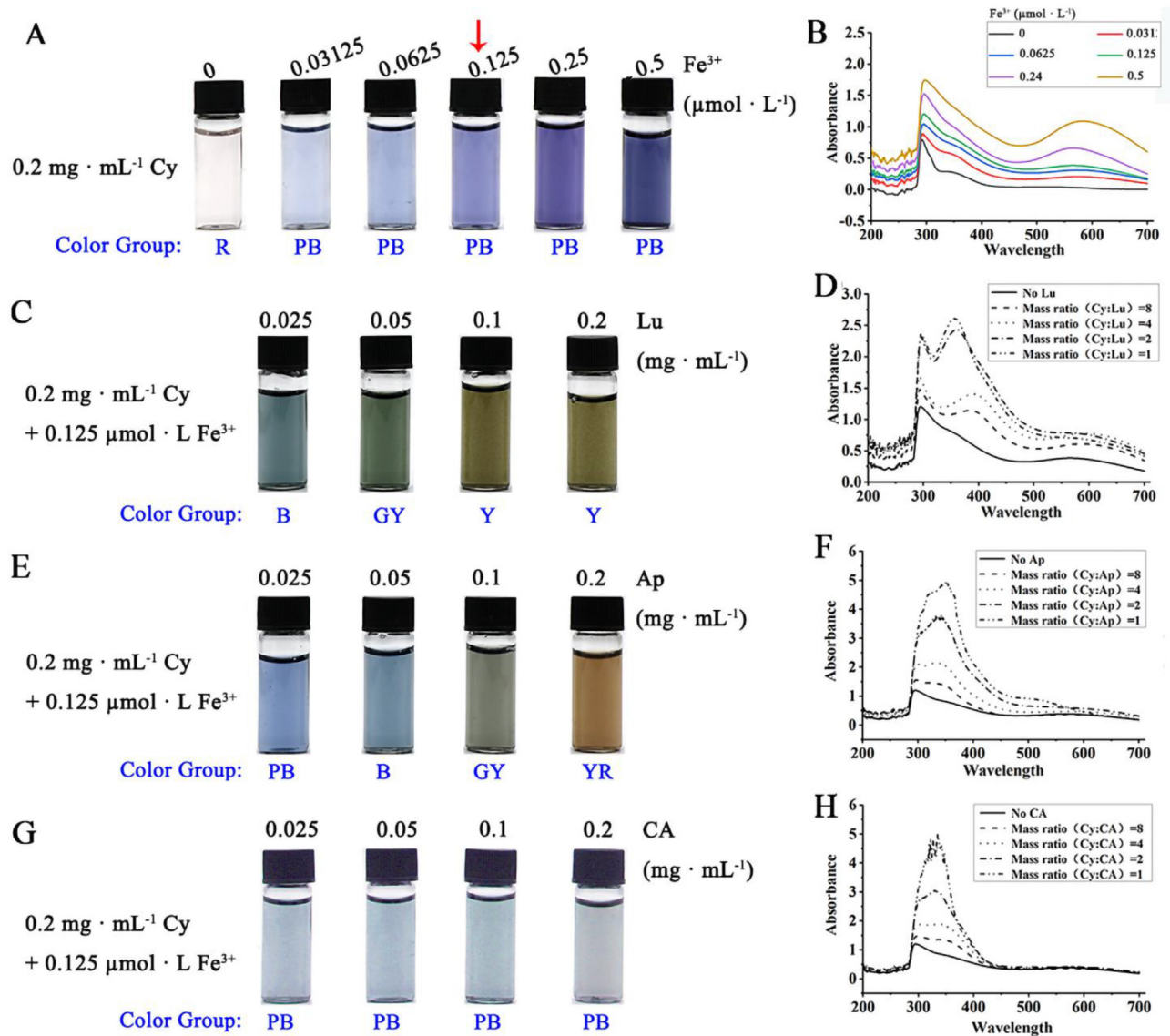


Fig. 5 Different copigment materials and concentrations affect the color of cyanidin-3-O-(6''-O-malonyl) glucoside with Fe^{3+} involved

A: Color change of cyanidin-3-O-(6''-O-malonyl) glucoside under different Fe^{3+} concentrations; B: Spectrum of cyanidin-3-O-(6''-O-malonyl) glucoside at different Fe^{3+} concentrations; C, E, G: The color changes of cyanidin-3-O-(6''-O-malonyl) glucoside at 0.2 $\text{mg} \cdot \text{mL}^{-1}$ and 0.125 $\mu\text{mol} \cdot \text{L}^{-1}$ of Fe^{3+} concentration, after adding different mass concentrations of luteolin (C), apigenin (E) and 3-caffeoylquinic acid (G); D, F, H: The spectral changes of cyanidin-3-O-(6''-O-malonyl) glucoside at 0.2 $\text{mg} \cdot \text{mL}^{-1}$ and 0.125 $\mu\text{mol} \cdot \text{L}^{-1}$ of Fe^{3+} concentration, after adding different mass concentrations of luteolin (D), apigenin (F), and 3-caffeoylquinic acid (H).

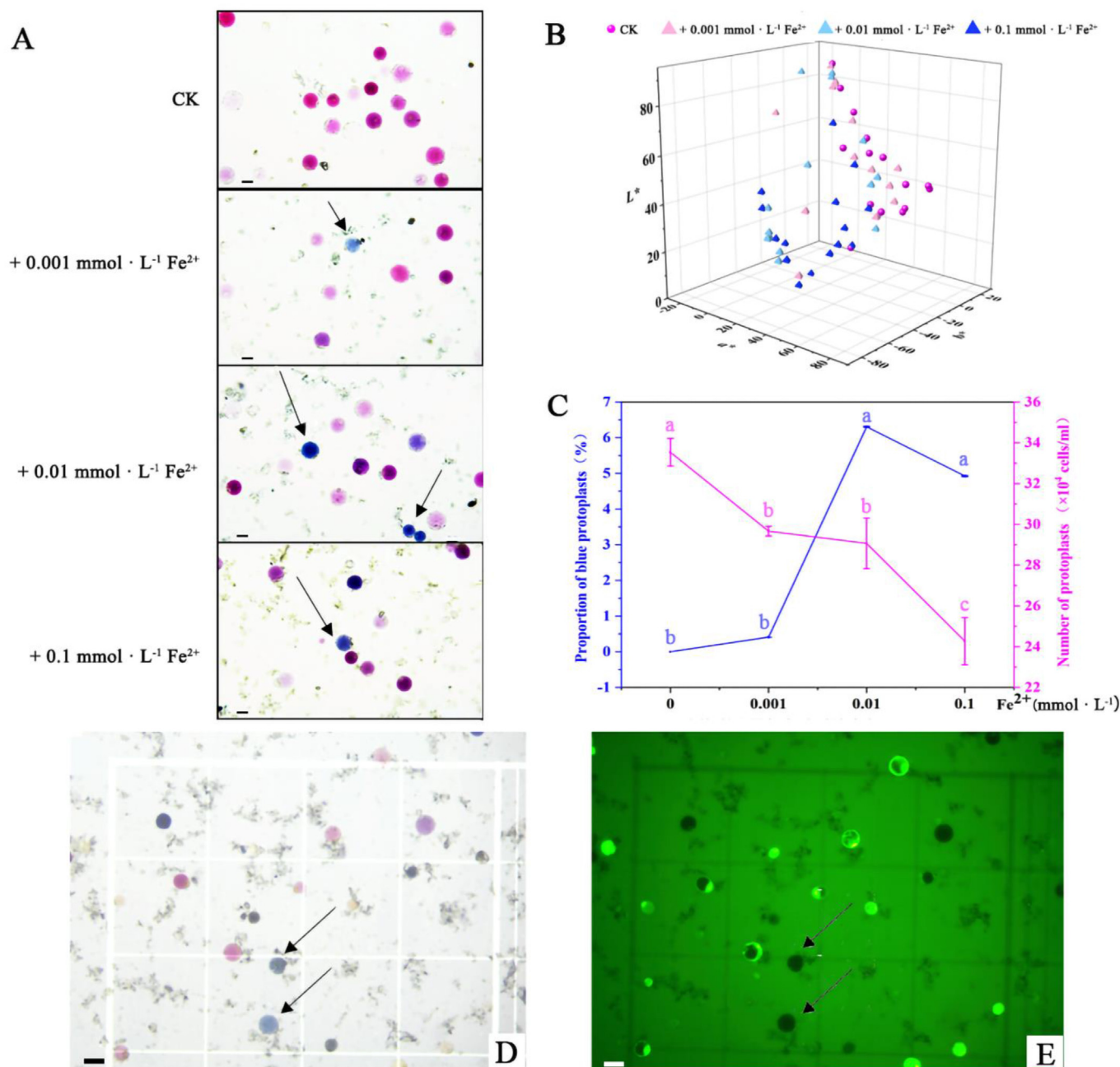


Fig. 6 Color phenotype of protoplasts of the chrysanthemum cultivar 'Wandai Fengguang' after adding Fe²⁺

A: The color appearance of protoplasts after adding different concentrations of Fe²⁺. The scale bars = 20 μm; B: The color phenotype of protoplasts after adding different concentrations of Fe²⁺; C: The results of adding different concentrations of Fe²⁺ to protoplasts that were resuspended in W5 buffer. The red line indicates the change in the total number of protoplasts with increasing Fe²⁺ concentration, while the blue line indicates the change in the number of protoplasts that turned blue with increasing Fe²⁺ concentration; D: FDA dyed protoplast under the bright light; E: FDA dyed protoplast under the ultraviolet light. The scale bars = 20 μm. Different lowercase letters indicate significant differences at $P < 0.05$ (Duncan's new multiple range test).

protoplasts showed a trend of first increasing and then decreasing (Fig. 6, C). Subsequently, the activity of protoplasts was measured, and it was found that the blue protoplasts became inviable (Fig. 6, D, E).

3.5.2. Ray florets appear blue in the presence of iron ions

After incubation in 50 mmol·L⁻¹ Fe²⁺ solution for 48 h, the ray florets of 'Wandai Fengguang' began to turn blue from the

base (Fig. 7, A). When the capitula of potted plants of 'Wandai Fengguang' were sprayed with 50 mmol·L⁻¹ Fe²⁺ solution, the tips of ray florets developed blue spots after 7 d (Fig. 7, B). Microscopic observation showed that the upper epidermis of the petals on the untreated plants was purplish red (Fig. 7, C), while the upper epidermis of the discolored zone at the base of the ray florets on the treated plants was darker purple and blue (Fig. 7, D). The upper epidermis of the blue spots at the ends of the ray

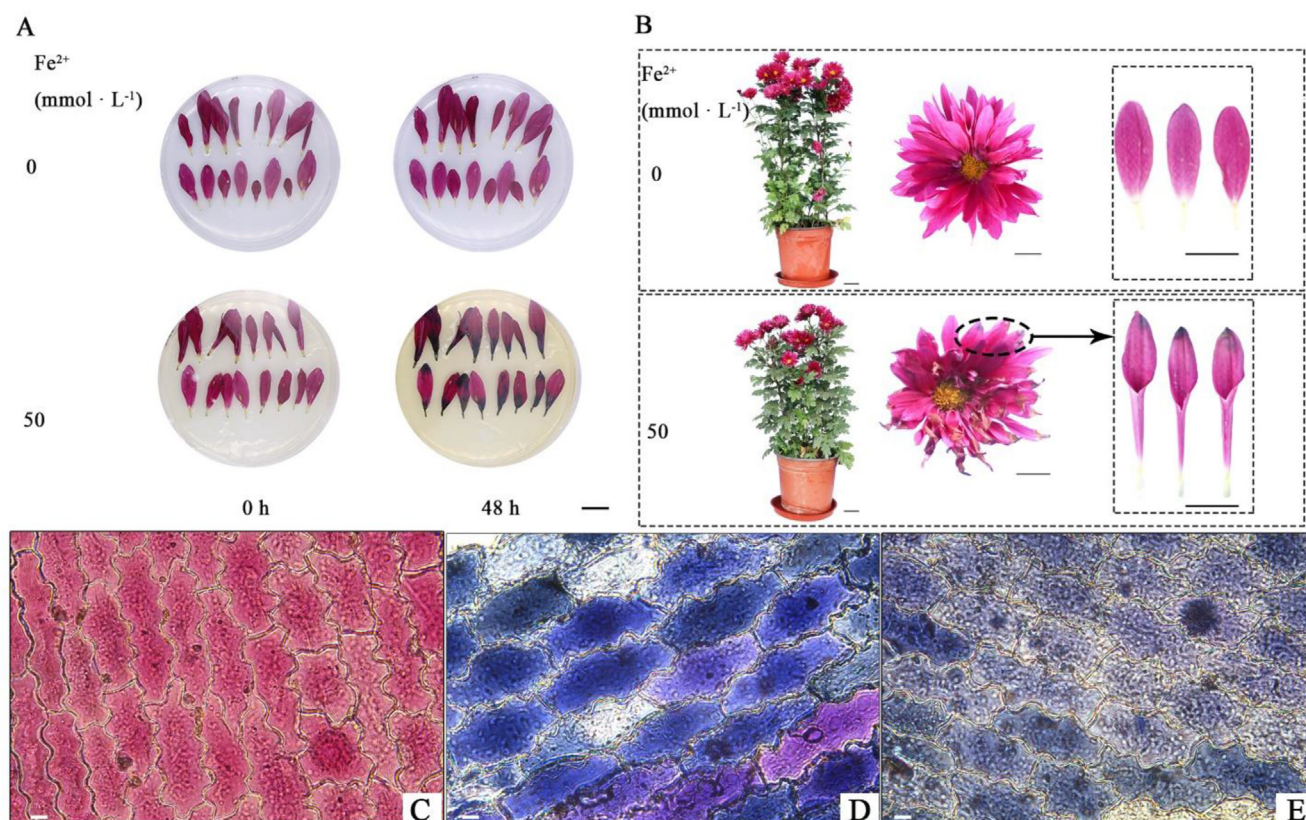


Fig. 7 Coloring of ray florets and potted flowers after the addition of Fe^{2+}

A: The color appearance of ray florets after adding different concentrations of Fe^{2+} , the scale bars = 1 cm; B: The color appearance of ray florets after spraying Fe^{2+} for potted flowers, the scale bars = 1 cm; C: The upper epidermis of the untreated petals. The scale bars = 10 μm ; D: The upper epidermis of the discolored zone at the base of ray florets. The scale bars = 10 μm ; E: The upper epidermis of the blue spots at the end of ray florets. The scale bars = 10 μm .

florets was mainly light purple and blue (Fig. 7, E), similar to the characteristics found in the protoplasts (Fig. 6).

4. Discussion

Blue flowers are one of the main objectives of ornamental plant breeding. World-famous flowers such as chrysanthemums, roses, and lilies all lack blue flowers because their petals only contain Cy content and lack Dp (Sun, 2010; Yamagishi and Nakatsuka, 2017; Xu et al., 2023). Therefore, extensive research has focused on reconstructing the Dp pathway. This molecular design breeding with multiple gene introductions is not only time-consuming and labor-intensive but also produces genetically modified flowers that are not sapphire blue (Brugliera et al., 2013; Noda et al., 2013, 2017). Although Cy causes most species to produce pink to red flowers, it causes a small number of species, such as *C. cyanus*, to produce sapphire blue flowers (Shiono et al., 2005; Takeda et al., 2005). The formation of sapphire blue metal complexes from Cy-based anthocyanins is facilitated by Fe^{3+} . However, earlier studies on ferric–anthocyanin chelates directed their focus toward the development of edible blue pigments and improving anthocyanin stability (Buchweitz et al., 2013; Sigurdson et al., 2016, 2017; Xie et al., 2018). There are no reports of the Cy pathway being used in the cultivation of blue flowers.

Hence, this study accomplished the first attempt to cultivate blue chrysanthemums by metal complexation through *in-vitro* and *in-vivo* experiments, making blue flower cultivation no longer limited to the Dp pathway. This provides a new approach for improving the color of flowers that accumulate Cy but cannot produce blue flowers.

4.1. Selection of the cultivar based on flower color phenotypes

Selecting cultivars based on flower color phenotypes is an important step in plant breeding. Carotenoids are stored in chromatids, and together with anthocyanins, determine color (Yu et al., 2023). However, carotenoids usually appear yellow and orange-red, which may influence any engineered colors (Brugliera et al., 2013; Lu et al., 2021; Zhou et al., 2021). Several studies have indicated that pink and red-purple petals contain low levels of carotenoids (Lu et al., 2021; Zhou et al., 2021). The 39 pink-purple chrysanthemums selected for this study were also found to be low in carotenoids, confirming previous findings.

The process used for selecting cultivars for blue flower breeding via the Cy pathway differs from that of the Dp pathway. This is due to the need to suppress the expression of the chrysanthemum native *F3'H* gene to achieve high Dp accumulation. Consequently, cultivars with low *F3'H* expression and light pink coloration are typically favored (Brugliera et al., 2013; Noda et al., 2017). In

contrast, the cultivation of blue chrysanthemum cultivars via the Cy pathway necessitates the accumulation of substantial amounts of Cy. Red-purple cultivars with a low L^* value and high a^* value are the most fitting, as their concentration of anthocyanins is inversely proportional to the L^* value and directly proportional to the a^* value (Zhou et al., 2021; Nguyen et al., 2022).

This paper provides a reference for selecting the right material for plants such as roses, lilies, and carnations that do not have blue flowers, but accumulate Cy, based on their floral color phenotypes. Cultivars with low brightness and high redness belonging to the red-purple group can be chosen as the materials.

4.2. Efficacy of the CB sample screening system

A prerequisite for quickly finding suitable receptor material is a stable screening system for cultivating blue flowers, whether through the Dp or Cy pathway. Through precursor feeding experiments of chrysanthemum petals with dihydromyricetin (DHM), a precursor of Dp, it was observed that 16 out of 53 cultivars exhibited a coloration change to mauve or purple after incubation (Brugliera et al., 2013). To evaluate the role of flower color genes, agroinfiltration-mediated transient expression was studied, and suitable host cultivars for stable transformation were identified in lilies and roses. The effects of the source of genes, their combination, and the host plant used in plant agroinfiltration were studied. After the experiment, five flower cultivars were found to be suitable for the accumulation of Dp, these being three rose cultivars and two lily cultivars out of a total of 30 and six cultivars, respectively (Zeinipour et al., 2018; Fallahpour et al., 2023). In this study, a stable screening system for CB samples was established using the chrysanthemum cultivar 'Wandai Fengguang' as a test material, and the optimum pH value of the buffer, the TAC added to the buffer, and the concentration range of Fe^{3+} were determined. Based on this system, 39 chrysanthemum cultivars were divided into five CB samples and 34 CN samples. The results of the *in-vivo* and *in-vitro* experiments were consistent and further illustrated the stability of this screening system. That is, the protoplasts of CB samples turned blue after ferrous iron supplementation, while CN samples did not turn blue. In addition, the ray florets of CB samples turned blue both on a ferrous supplemented medium and after ferrous spraying. This is the initial method established to screen host materials based on the Cy pathway, and lays the foundation for breeding blue flowers using the Cy pathway.

4.3. Effect of the pH on ferric–anthocyanin chelate

In this paper, it was found that the color difference of flavonoid extracts with different buffer pH values was not significant after the addition of iron ions. This finding is consistent with previous findings that anthocyanins can form blue anthocyanin–metal chelates with the involvement of metal ions over a wide pH range (Sigurdson and Giusti, 2014; Sigurdson et al., 2016). It is assumed that although the pH of the solution can lead to different colorations through affecting the structure of anthocyanins, the combination with metal ions can shift and stabilize all the different anthocyanin structures toward the structure of the blue anionized quinone-type bases. In the present study, the optimal pH value of 5.5 was screened based on the bathochromic shift and the hyperchromic shift after the addition of Fe^{3+} , which was similar to that reported by Sigurdson et al. (2017). Based on the pH

value (average pH 5.6) in chrysanthemum vacuoles measured by Noda et al. (2017), it is speculated that the pH conditions in chrysanthemum vacuoles are conducive to the formation of anthocyanin–iron chelates.

4.4. Effect of the ratio of TAC to Fe^{3+} on ferric–anthocyanin chelate

To obtain a blue color, no more Fe^{3+} should be added, but the ratio of TAC to Fe^{3+} should be adjusted to within the appropriate range. This is in accord with previous studies. For example, cornflower only showed blue color when the ratio of Cy 3-O-(6-O-succinylglucoside)-5-O-glucoside, apigenin 7-O-glucuronide-4'-O-(6-O-malonylglucoside), and Fe^{3+} was 6:6:1 for the formation of blue supramolecular pigments (Takeda et al., 2005). The color of Dp 3-O-rutinoside in tulips changed from purple to violet, then to blue and finally to brown with the addition of increasing concentrations of Fe^{3+} (0.1, 0.5, 1.0, 3.0, 5.0, and 10.0 mmol·L⁻¹) (Shoji et al., 2006). *Artemisia grandiflora* was able to reproduce the blue color with the addition of a certain proportion of Cy 3-O-[(6-O-malonyl-2-O-β-D-xylopyranosyl)-β-D-glucopyranoside]-7-O-β-D-glucopyranoside, kaempferol derivatives, Fe^{3+} , and Mg^{2+} . However, when excess Fe^{3+} was added, a blue-black color appeared (Yoshida et al., 2006). Due to the difficulty of separation and purification to obtain the monomers of anthocyanins and flavonoids in chrysanthemum, this paper focused only on the effect of iron ions on the color of chrysanthemum flavonoid extracts, and circular dichroism spectra were not performed. Therefore, it was not possible to determine whether the metal complexes belonged to the stoichiometric metal–pigment–copigment complexes or the non-stoichiometric metal–pigment–copigment complexes. This part of the experiment will be further improved in future work.

4.5. Effect of the types and concentration of copigments on ferric–anthocyanin chelate

In this study, chlorogenic acid could not cause Cy to turn blue or produce hyperchromic effects. This is because the core of anthocyanin comprises a π -conjugation system that is entirely off-domain and rapidly forms supramolecular assemblies with other pigments and cofactors, enabling it to undergo color changes upon the addition of copigments (Trouillas et al., 2016). However, the varying structures of copigments cause their complex effects on anthocyanin to differ. Flavonoids possess a similar planar structure to anthocyanins, and therefore, they have a stronger complexing effect on anthocyanin compared to hydroxycinnamic acid (Houghton et al., 2021). The results of this study concluded that luteolin and apigenin exhibited a more potent hyperchromic effect and bathochromic shifting compared to chlorogenic acid, which belongs to hydroxycinnamic acid. The primary flavonoids in chrysanthemum, luteolin and apigenin, provided the Cy in chrysanthemum with the potential to turn blue when Fe^{3+} was added. It is suggested that the luteolin's B-ring neighboring hydroxyl group enhances its ability to bind to Fe^{3+} , resulting in an increased color change of the flavonoid extract solution.

It was also established that the ratio of TAC to TLC in the flavonoid fraction was the key reason for the decision to categorize chrysanthemum cultivars into CB and CN samples. Based on the analysis of flavonoid fractions from five CB samples and eight CN samples, it was concluded that TAC: TLC ≥ 2.25 is a

prerequisite for a chrysanthemum variety to be classified as a CB sample. However, only five varieties were used for flavonoid fractions in this study, and it is believed that the TAC: TLC ratios would be more accurate if more CB samples were analyzed for flavonoids. Moreover, this paper confirms the conclusion that the ratio of TAC to TLC affects the color of anthocyanins with the involvement of iron ions using standards. This is the first time that the conditions of flavonoid fractions of CB samples have been elucidated. Furthermore, this study provides a clear direction for the future screening of more CB samples in chrysanthemums, roses, lilies, and carnations.

4.6. Feasibility of cultivating blue flowers based on the Cy pathway

Protoplasts from the CB samples ‘Wandai Fengguang’, ‘Ziyan Fanfei’, and ‘Dongli Zijiao’ demonstrated blue coloration in the presence of Fe^{3+} . It was observed that the base of the ray florets of ‘Wandai Fengguang’ had a blue coloration when placed on Fe^{2+} medium. Moreover, visible blue spots were observed on the ray florets of ‘Wandai Fengguang’ in pots following the application of Fe^{2+} . The occurrence of these phenomena confirms that the flavonoid components of the petals of CB samples can be successfully dyed blue with the addition of iron ions *in vivo*. Breeding blue flowers based on the Cy pathway does not need to consider the flavonoid fraction of chrysanthemum itself, and using screened CB samples as test material only changes the intracellular iron ion concentration. This pathway is simpler than the Dp pathway.

Iron, which can only enter cells in its reduced form (Fe^{2+}), can only be stored or bound to carrier proteins in the trivalent iron state (Fe^{3+}) (Millonig et al., 2010). The excessive amount of free Fe^{2+} is oxidized to trivalent iron in the presence of hydrogen peroxide metabolized by the cell via the Fenton reaction, leading to the production of dangerous hydroxyl radicals that result in the death of cells (Kell and Pretorius, 2014; Camaschella et al., 2020). Thus, as the concentration of Fe^{2+} increased, the number of protoplasts decreased, and the blue protoplasts exhibited a pattern of initially rising and then falling. This paper also revealed that the protoplasts that turned blue were all inactivated. It was speculated that Fe^{2+} was oxidized to Fe^{3+} by the Fenton reaction. This formed hydroxyl radicals and led to the inactivation of the cells, which may be the cause of this phenomenon.

Investigations regarding the influence of metal ions on flower color have primarily focused on *Hydrangea macrophylla*. The addition of aluminum ions to this plant led to the transformation of sepals from pink to blue while leaving the growth of sepals unaffected (Yuan et al., 2023). This study, however, involved the application of Fe^{2+} to ‘Wandai Fengguang’ flowers, which caused the ray florets on the inflorescences to appear wilted and scorched on their tips. Therefore, it is imperative to find a way to accumulate sufficient iron ions in the petals without affecting their growth and development. Previous studies have explored methods to increase the iron ion concentration in tissue vacuoles through transgenic means (Narayanan et al., 2015; Sheraz et al., 2021). This suggests that breeding blue flowers with higher iron ion concentration in their petal vacuoles through molecular breeding is a feasible possibility to pursue. This is the first attempt to breed blue chrysanthemum in the Cy pathway. The authors will continue to search for ways to increase the iron ion

concentration in the petal vacuoles of chrysanthemums without affecting their growth and development in order to breed chrysanthemum cultivars with a sapphire blue color and provide valuable references for other plants.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.hpj.2023.10.002>.

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