Copigmentation gives bluer flowers on transgenic torenia plants with the antisense dihydroflavonol-4-reductase gene

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Abstract

When anthocyanins in plants make complexes with copigments such as flavones or flavonols (copigmentation), the visible absorption maximum of the flowers is shifted so that it becomes longer: that is, the flowers look bluer. In an earlier study, our group reported the modification of flower color in torenia (Torenia fournieri Lind.) by re-introduction of the dihydroflavonol-4-reductase (DFR) gene or the chalcone synthase (CHS) gene. Our initial observation of torenia transformants was that plants with the antisense DFR gene produced bluer flowers than plants with the antisense CHS gene. In the present study we found that inactivation of the DFR gene by genetic transformation caused the accumulation of flavones — possible copigments — and that the resulting copigmentation likely to make the torenia flowers bluer. This method could be applied to other plant species to produce bluer flowers. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Anthocyanins are the most important pigments for determining flower color. There are six main anthocyanidins (aglycons of anthocyanins): pelargonidin; cyanidin; peonidin; delphinidin; petunidin; and malvidin. As the number of hydroxyl groups in the B-ring increases, the visible absorption maximum shifts to become longer, so that the flower looks bluer. Anthocyanidins are normally modified with glycosylation and acylation to produce a wider variety of anthocyanins. The flower colors produced by anthocyanins are influenced by the structures of the pigments, the vacuolar pH, the quantities of copigments present and the epidermal cell shapes [1]. When anthocyanins make complexes with copigments such as flavones or flavonols (by copigmentation), the visible absorption maximum shifts to become longer (bathochromic shift) [2,3]. Forkmann [4] pointed out that the copigmentation present in a flower is one of the most important factors in determining its blueness.

In recent years, progress has been made in the use of genetic transformation to modify flower color, which is one of the most important characteristics of ornamental plants [1,5–8]. Florigene Ltd., and Suntory Ltd., have created a transgenic blue carnation and have marketed it in Australia and Japan [1]. This carnation contains the petunia flavonoid 3', 5'-hydroxylase gene, and the resultant production of delphinidin makes its flowers bluish. Apart from the production of delphinidin, raising of the vacuolar pH [9], acylation of aglycon and intramolecular or intermolecular stacking (includ-
ing copigmentation) [10] are presumed to be important factors in the production of blue flowers.

Genetic transformation can be done by either introducing and expressing a new gene or reintroducing an existing gene in sense or antisense orientation to inactivate an endogenous gene [11]. In several ornamental plants, genetic transformation has been used to modify flower color by the suppression of the chalcone synthase (CHS) or dihydroflavonol-4-reductase (DFR) genes that encode enzymes in the biosynthetic pathway of anthocyanins [12–17]. Our group previously reported the modification of flower color in toreonia (Torenia fournieri Lind., family Scrophulariaceae) by reintroduction of DFR or CHS genes [18]. Our initial observation of torenia transplants was that plants with the antisense DFR gene produced bluer flowers than plants with the antisense CHS gene (Fig. 1). The study reported here is the first to show that a copigmentation effect caused by the accumulation of flavones in DFR-introduced plants likely to be the reason for the bluing phenomenon.

2. Materials and methods

2.1. Plant materials

We used Torenia fournieri Lind., plants transformed with an antisense CHS gene (411-3) or an antisense DFR gene (416-20) obtained from a clonal laboratory line (Crown Violet) selected from ‘Crown Mix’. The Crown Violet was used as a wild type plant. These transgenic plants were obtained in a previous study [18]. Both transgenic plants represented typical flower colors of their groups: the flowers of 411-3 appeared reddish and those of 416-20 appeared bluish.

2.2. Observation of epidermal cells and measurement of absorption spectra

The colored parts of the fully expanded petals were cut, mounted in McIlvaine's citrate-phosphate buffer solution (pH 5.4), and viewed under a microscope. We peeled the epidermis of the colored parts of the fully expanded petals so that we could measure the visible absorption with a spectrophotometer (UV-240, Shimadzu, Kyoto, Japan). We also crushed the colored parts of the fully expanded petals with a pestle and mortar. We then placed a drop of the resultant juice on a glass slide with a cover slip to measure the visible absorption with the spectrophotometer.

2.3. Identification of anthocyanins and flavones

To identify the anthocyanins and flavones, we obtained an extract from about 600 g of torenia petals by placing them in 3 l 50% acetonitrile and 3% trifluoroacetic acid solution for 3 h. The residue was re-extracted twice with 2.5 l 50% acetonitrile and 0.2% trifluoroacetic acid solution. The rotary-evaporated extract was applied to an XAD-7 column and eluted with 5, 10, 20, 30, 40, 50 and 80% acetonitrile (in 0.2% trifluoroacetic acid). Five anthocyanins were detected in the 20% acetonitrile fraction, but flavones were not detected. The 40% acetonitrile fraction contained three major flavones and traces of anthocyanins. The eluates of both were further purified by ODS-HPLC in a Develosil ODS-HG-5 column (20 × 250 mm, Nomura Chemical, Seto, Japan) with 10–25% acetonitrile (in 0.5% trifluoroacetic acid) at 40°C at a flow rate of 5.0 ml/min. We used FABMS and 1H NMR (JNM-A600, JEOL, Tokyo, Japan) to identify the five purified anthocyanins and three purified flavones.

2.4. Extraction and quantification of anthocyanins and flavones

Fresh torenia petals (1.0 g fresh weight for each sample) were incubated for 20 h with 50 ml 5% (v/v) acetic acid in methanol or 50 ml methanol to extract the anthocyanins or flavones, respectively. The extract was rotary-evaporated to near-dryness. Samples for anthocyanin extraction were dissolved in 20% solvent A (25% acetonitrile, 20% acetic acid and 1.5% phosphoric acid) in solvent B (1.5% phosphoric acid) for HPLC analysis. Samples for flavone extraction were dissolved in 10 ml of water and washed with petroleum ether three times. The washed water fraction was rotary-evaporated to near-dryness and dissolved with solvent D (20% acetonitrile and 0.2% phosphoric acid) for HPLC analysis. The anthocyanin samples were analyzed by HPLC in a LiChrospher 100 RP-18 column (4 × 250 mm, Kanto Chemical, Tokyo) with a linear-gradient solvent system of 20–100% of solvent A in solvent B for 40 min at 40°C at a
flow rate of 1.0 ml/min. The flavone samples were analyzed by HPLC in the same column with a linear-gradient solvent system of 0–100% of solvent C (40% acetonitrile and 0.2% phosphoric acid) in solvent D for 40 min at 40°C at a flow rate of 1.0 ml/min. The eluted components were monitored at 530 nm for anthocyanins and 340 nm for flavones. The relative quantities of an-

Fig. 1. Flower colors of plants used in the experiment. (A) A violet line selected from ‘Crown Mix’ (Crown Violet) and used as a wild type plant. (B) Typical reddish flower of a plant (411-3) transformed with an antisense CHS gene derived from the Crown Violet. (C) Typical bluish flower of a plant (416-20) transformed with an antisense DFR gene derived from the Crown Violet.
thocyanins and flavones among the samples were determined from the peak areas of the chromatogram.

2.5. Copigmentation test

To examine the anthocyanin–flavone copigmentation, we dissolved the dried 20% acetonitrile fraction (containing anthocyanins) and the dried 40% fraction (containing flavones) in McIlvaine’s citrate-phosphate buffer solution (pH 5.4) and then used the solutions to make mixed samples with different final concentrations. (The derivation of these fractions was described above in Section 2.3.) The visible absorbance of each mixed solution was measured 8 h after mixing.

3. Results and discussion

3.1. Observation of epidermal cells and measurement of absorption spectra

The flower colors might be influenced by the structure of epidermal cells and distribution of pigments. In the transgenic plants, the structure of the epidermal cells, the distribution of the vacuoles and the distribution of pigments in the vacuoles remained the same as in the wild type plants, regardless of any differences in the intensity and hue of the vacuole color (Fig. 2). It seemed that the structure of the cells and the distribution pattern of pigments was not affected the modified flower colors.

The absorbance maximum ($\lambda_{\text{max}}$) of the intact petals was about 545 nm for the wild type plant, 545 nm for plants transformed with the antisense CHS gene (411-3) and 575 nm for plants transformed with the antisense DFR gene (416-20) (Fig. 3a). The $\lambda_{\text{max}}$ of the 411-3 plants was almost the same as that of the wild type plants, but that of the 416-20 plants had shifted to become about 30 nm longer. The $\lambda_{\text{max}}$ of the freshly squeezed juice from the petals was about 540 nm for the wild type plants, 540 nm for plants transformed with the antisense CHS gene (411-3) and 580 nm for plants transformed with the antisense DFR gene.
These values were almost same as the values obtained with the intact petals. The \( \lambda \) max of 416-20 shifted to become about 40 nm longer than that of the wild type plants and 411-3. These results suggested that the reddish appearance of the flowers of 411-3 was a less intense version of the colour of the wild type plants, and that these two types of plants had a similar basic hue. In contrast, the bluish flowers of 416-20 were a different color from those of the wild type and 411-3 plants.

The flower colors produced by anthocyanins are influenced by the vacuole pH [1]. Higher vacuole pH might lead to bluer colors. The pH values for the squeezed juices from the wild type, 411-3 and 416-20 plants were all about 5.4. The colors of both the intact petals and the juice of the petals were almost the same for each of the plants (wild type and both transgenic plants). This suggested that the pH values of both the intact vacuoles and the squeezed juice of the petals were likely to be the same in all the plants. Since the pH values of the petal juices of all the plants were indeed found to be the same (at about 5.4), we considered that the pH values in the vacuoles were the same in the wild type torenia and the both transgenic torenias. This result suggested that the bluer flowers of transgenic torenia were not caused by higher vacuole pH.

3.2. Identification and quantification of anthocyanins and flavones

The flower colors are influenced by the varieties and the quantities of anthocyanins and copigments. The five anthocyanins detected in the torenia were identified as delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, petunidin 3,5-diglucoside, peonidin 3,5-diglucoside and malvidin 3,5-diglucoside at peaks 1, 2, 3, 4 and 5, respectively (Fig. 4, left). The three major flavones were identified as luteolin 7-glucoside, luteolin 7-glucuronide and apigenin 7-glucuronide at peaks 1, 2 and 3, respectively (Fig. 4, right).

There were five anthocyanin peaks in the wild type plants (Fig. 4, left). Both of the transformants showed reduced levels of anthocyanins but the same peaks. The 411-3 plants contained a small amount of peonidin 3,5-diglucoside and a large amount of malvidin 3,5-diglucoside, and the 416-20 plants contained mainly malvidin 3,5-diglucoside. Although the anthocyanin profiles of 411-3 and 416-20 differed slightly, this difference was not the reason for the bluing phenomenon, since malvidin 3,5-diglucoside, a major anthocyanin of the bluish transgenic line, is pinkish at pH 5.4 (Data not shown.). The total quantity of anthocyanin in 411-3 was 15% of that of the wild type plants, and the total quantity of anthocyanin in 416-20 was 14% of that of the wild type plants.

There were three major and several minor peaks for flavones in the wild type plants (Fig. 4, right).
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Fig. 4. HPLC analysis of anthocyanins and flavones. There were five anthocyanin peaks in the wild type plants. Both of the transformants showed the same peaks but reduced levels of anthocyanins. The 411-3 plants showed mainly the last two peaks, and the 416-20 plants showed mainly the last peak. There were three major and several minor peaks for flavones in the wild type plants. The retention times of the peaks detected for both transformants were the same as for the wild type plants, but the total quantity of flavones in the 411-3 plants was reduced to 34% of that of the wild type plants, and that in the 416-20 plants was increased to 151% of that of the wild type plants.

The variety of flavones present was almost the same among the three lines. The total amount of flavones present in 411-3 was reduced to 34% of that found in the wild type, but in 416-20 it was increased to 151% of that present in the wild type. The decrease in the total amount of anthocyanins and the increase in the total amount of flavones in 416-20 may have elevated the copigment to pigment ratio and caused copigmentation in the bluish flowers.

Flavones are synthesized from flavanones, which are intermediates in the anthocyanin pathway (Fig. 5). A reduction in CHS activity might reduce the anthocyanin content and flavone content simultaneously, because CHS is located in the upper stream of the anthocyanin pathway. A reduction in DFR activity might reduce the anthocyanin content but increase the flavone content, because DFR catalyzes the lower stream of the anthocyanin pathway. Therefore, one would expect that only the plants with the antisense DFR gene would have an increased amount of flavones.

3.3. Copigmentation test

Copigmentation test was performed to examine the effect of flavones for the bluing phenomenon. As the density of the flavone fraction increased, the color of the anthocyanin–flavone mixed solution became deeper and bluer (Fig. 6). The \( \lambda \) max was 540 nm when the flavone density was 0.1 mg/ml (anthocyanin density 2.5 mg/ml): this was the almost same \( \lambda \) max seen in the intact petals and squeezed petal juice of the wild type plants and 411-3 plants. The \( \lambda \) max was shifted longer to

Fig. 5. Biosynthetic pathway of anthocyanins. Flavones are synthesized from flavanones, which are intermediates in the anthocyanin pathway. A reduction in CHS activity could reduce the quantities of both anthocyanins and flavones present. A reduction in DFR activity, however, could reduce only the quantity of anthocyanins and increase the quantity of flavones. CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase.
Fig. 6. Copigmentation test with anthocyanins and flavones. The anthocyanin fraction and the flavone fraction extracted from the torenia plants were dissolved in McIlvaine’s citrate-phosphate buffer solution (pH 5.4) and then mixed together in various ratios. The visible absorbance of the mixed solutions was measured 8 h after mixing. As the density of the flavone fraction increased, the color of the anthocyanin–flavone solution became deeper and bluer. The \( \lambda \) max was 540 nm when the flavone density was 0.1 mg/ml (anthocyanin density 2.5 mg/ml): the same value found in the intact petals and squeezed petal juice of the wild type plants. The \( \lambda \) max shifted longer to 570 nm when the flavone density reached 0.75 mg/ml (anthocyanin density 2.5 mg/ml): the same value found in the intact petals and squeezed petal juice of the bluish transgenic plants (416-20). It seems that the copigmentation phenomenon occurred in flowers of transgenic plants with the antisense DFR gene, making the flowers bluer than those of the wild type plants and transgenic plants with the antisense CHS gene. Baranac et al. [19] demonstrated a shift in the absorption maximum of nearly 30 nm when 0.9-mM apigenin 7-glucoside was mixed with 0.3-mM malvidin 3,5-diglucoside in a buffer solution of pH 3.65. They suggested that the presence of a sugar molecule in position 7 caused the large bathochromic shift, because apigenin alone, which does not have a sugar molecule in position 7, does not show a bathochromic shift. The fact that the major flavones of torenia — luteolin 7-glucoside, luteolin 7-glucuronide and apigenin 7-glucuronide — have sugar molecules in position 7 suggests that these molecules contribute to the bluing effect in transgenic torenia plants with the antisense DFR gene.

4. Conclusion

Molecular breeding of blue flowers has been achieved in carnations by the production of delphinidin. Tanaka et al. [1] have pointed out that
the acylation of aglycon, raising the vacuolar pH and intramolecular or intermolecular stacking (including copigmentation) would be other valuable steps in the production of blue flowers. Holton et al. [20] reported that the quantity of flavonols (possible copigments) could be modified by introducing a flavonol synthase (FLS) gene. Control of the FLS gene would be one way of changing the quantity of copigments present and possibly producing blue flowers, but there is no report on the production of bluer flowers by this strategy. We demonstrated, instead, that another strategy — inactivation of the DFR gene by re-introducing it — accumulated flavones and/or flavonols as possible copigments, and that the resulting copigmentation made flowers bluer. This method could be applied to other plant species in the future to produce bluer flowers.

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