Additional betalain accumulation by genetic engineering leads to a novel flower color in lisianthus (*Eustoma grandiflorum*)

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Abstract Betalains, comprising violet betacyanins and yellow betaxanthins, are pigments found in plants belonging to the order Caryophyllales. In this study, we induced the accumulation of betalains in ornamental lisianthus (*Eustoma grandiflorum*) by genetic engineering. Three betalain biosynthetic genes encoding CYP76AD1, dihydroxyphenylalanine (DOPA) 4,5-dioxygenase (DOD), and cyclo-DOPA 5-O-glucosyltransferase (5GT) were expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in lisianthus, in which anthocyanin pigments are responsible for the pink flower color. During the selection process on hygromycin-containing media, some shoots with red leaves were obtained. However, most red-colored shoots were suppressed root induction and incapable of further growth. Only clone #1 successfully acclimatized and bloomed, producing pinkish-red flowers, with a slightly greater intensity of red color than that in wild-type flowers. T1 plants derived from clone #1 segregated into five typical flower color phenotypes: wine red, bright pink, pale pink, pale yellow, and salmon pink. Among these, line #1-1 showed high expression levels of all three transgenes and exhibited a novel wine-red flower color. In the flower petals of line #1-1, abundant betacyanins and low-level betaxanthins were coexistent with anthocyanins. In other lines, differences in the relative accumulation of betalain and anthocyanin pigments resulted in flower color variations, as described above. Thus, this study is the first to successfully produce novel flower color varieties in ornamental plants by controlling betalain accumulation through genetic engineering.

Key words: anthocyanins, betacyanins, flower color, lisianthus, transgenic plants.

Introduction

Plant pigments are responsible for colors of reproductive organs, such as flowers and fruits, as well as that of vegetative tissues. Four pigment classes have been reported in the plant kingdom, including anthocyanins (flavonoids), carotenoids, chlorophylls, and betalains (Tanaka et al. 2008). The former three pigments are biosynthesized and accumulated in almost all plants. On the other hand, betalains, comprising yellow betaxanthins and red–violet betacyanins, are found only in some species belonging to the order Caryophyllales (Gandia-Herrero and Garcia-Carmona 2013). Although betalains can act as a substitute for the ubiquitous anthocyanin pigments, anthocyanins and betalains generally exhibit mutually exclusive distribution in plants (Timoneda et al. 2019).

Enzymatic genes involved in the betalain biosynthetic pathway have been identified in the last decade (Figure 1). Cytochrome P450-type enzyme, CYP76AD1, converts tyrosine into L-dihydroxyphenylalanine (L-DOPA) 4,5-dioxygenase (DOD), and cyclo-DOPA 5-O-glucosyltransferase (5GT) were expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in lisianthus, in which anthocyanin pigments are responsible for the pink flower color. During the selection process on hygromycin-containing media, some shoots with red leaves were obtained. However, most red-colored shoots were suppressed root induction and incapable of further growth. Only clone #1 successfully acclimatized and bloomed, producing pinkish-red flowers, with a slightly greater intensity of red color than that in wild-type flowers. T1 plants derived from clone #1 segregated into five typical flower color phenotypes: wine red, bright pink, pale pink, pale yellow, and salmon pink. Among these, line #1-1 showed high expression levels of all three transgenes and exhibited a novel wine-red flower color. In the flower petals of line #1-1, abundant betacyanins and low-level betaxanthins were coexistent with anthocyanins. In other lines, differences in the relative accumulation of betalain and anthocyanin pigments resulted in flower color variations, as described above. Thus, this study is the first to successfully produce novel flower color varieties in ornamental plants by controlling betalain accumulation through genetic engineering.

Abbreviations: 35Sp, cauliflower mosaic virus 35S promoter; 5GT, cyclo-DOPA 5-O-glucosyltransferase; BAP, 6-benzylaminopurine; CaMV, cauliflower mosaic virus; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; DOD, DOPA 4,5-dioxygenase; DOPA, dihydroxyphenylalanine; HPT, hygromycin phosphotransferase; RT-PCR, reverse transcription polymerase chain reaction.

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The spontaneous conjugation of betalamic acid with amines or cyclo-DOPA derivatives results in the formation of betaxanthins or betacyanins, respectively (Strack et al. 2003). The betacyanin biosynthetic pathway also progresses via an alternative glycosylation route, leading to the conversion of betanidin into betanin. Moreover, cyclo-DOPA 5-O-glucosyltransferase (5GT) is involved in the glycosylation of betacyanin (Sasaki et al. 2005). The expression of betalain-related genes is regulated by a conserved anthocyanin-regulating transcription factor complex, namely, MYB-bHLH-WDR (MBW); in beet (Beta vulgaris), BvMYB1 has been shown to comprise the MBW complex (Hatlestad et al. 2015).

In some non-Caryophyllales species, transgenic betalain-accumulating plants have been produced by the heterologous-expression of betalain biosynthetic genes. For example, transgenic Arabidopsis thaliana plants expressing the DOD from fungus Amanita muscaria produced betaxanthins and betacyanins when supplied with the substrate L-DOPA (Harris et al. 2012). Transgenic tobacco BY2 cells expressing both DOD and tyrosinase genes from shiitake (Lentinula edodes) accumulated betaxanthins without the exogenous application of L-DOPA (Nakatsu et al. 2013). Polturak et al. (2016) reported that the heterologous-expressions of CYP76AD1, DOD, and 5GT induced the accumulation of betacyanins in whole tobacco tissues. Moreover, yellow betaxanthins were produced in transgenic tobacco and Arabidopsis plants expressing CYP76AD5 or CYP76AD6 together with DOD (Polturak et al. 2016; Sunnadeniya et al. 2016). In horticultural Solanaceae plants, a similar genetic engineering approach induced de novo betalain accumulation in whole tissues of transgenic potato, eggplant, tomato, and petunia (Polturak et al. 2017). However, to our knowledge, there are no reports on the metabolic engineering of betalains in any species except for Arabidopsis and Solanaceae plants and in ornamental plants except for petunia.

Lisianthus (Eustoma grandiflorum [Raf.] Shinn.), an ornamental plant belonging to the Gentianaceae family, is becoming an increasingly popular cut flower because of its large flowers, long stems, and extended vase life (Dole and Wilkins 2005). In Japan, lisianthus was the fifth highest contributor to the total wholesale value of cut flowers in 2019. Most lisianthus cultivars distributed worldwide are bred by Japanese nurseries. Lisianthus may be divided into two groups: single-flowering and double-flowering (Nau 2011). Flowers of different lisianthus cultivars accumulate anthocyanins or carotenoids in their petals, thus exhibiting various colors, including white, pale yellow, pink, and purple (Liu et al. 2013; Uddin et al. 2004). Different flower color varieties are also produced by conventional breeding and molecular biology tools (Handa and Deroles 2001). Previous metabolic engineering studies in lisianthus have targeted mainly the anthocyanin biosynthetic pathway. The introduction of the antisense chalcone synthase (CHS) in lisianthus changed the flower color from purple to white...
or patterned purple with small white streaks (Deroles et al. 1998). By contrast, transgenic lisianthus expressing the antisense flavonol synthase (FLS) produced flowers with greater intensity of red color than the wild-type lisianthus (Nielsen et al. 2002). However, to date, the modification of lisianthus flower color via the genetic engineering of the betalain biosynthetic pathway has not been reported.

Here, we produced transgenic lisianthus lines heterologously-expressed CYP76AD1, DOD, and 5GT, which encode betalain biosynthetic enzymes. The resultant progeny showed several flower color phenotypes, which were associated with different levels of betalain and anthocyanin accumulation. Our data suggest that altering betalain accumulation by genetic engineering could be used to increase the range of flower color in lisianthus and other floricultural plants.

Materials and methods

Plant material

Seeds of lisianthus (E. grandiflorum cv. Light pink sum) were purchased from Fukukaen Nursery and Bulb Co. (Nagoya, Japan), and then aseptically sown on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1% (w/v) sucrose and 0.2% (w/v) gellan gum. The plates were incubated at 25°C under a 16-h light/8-h dark photoperiod.

Preparation of Agrobacterium

The best CYP76AD1 open reading frame (ORF) (HQ656023) was synthesized by Eurofins Genomics (Tokyo, Japan). Additionally, plasmids containing the cloned cdNA of DOD (Sasaki et al. 2009) and 5GT (Sasaki et al. 2005) from Mirabilis jalapa were provided by Professor Yoshihiro Ozeki (Tokyo University of Agriculture and Technology). The ORFs of these three genes, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and Arabidopsis heat shock protein terminator (Nagaya et al. 2010), were cloned in-tandem into the binary vector pSMAH704, which was converted from the bar to the hygromycin phosphotransferase (HPT) in pSMAB704 (Igasaki et al. 2002), to generate the pShyg-35SpCYP76AD1-35SpDOD-35Sp5GT plasmid (Figure 2). The pShyg-35SpintronGUS construct was used as a vector control. These binary vectors were transformed into Agrobacterium tumefaciens strain EHA101 by electroporation, and the transformed Agrobacterium cells were selected on solid LB medium, supplemented with 200 mg l⁻¹ spectinomycin, 30 mg l⁻¹ kanamycin, and 25 mg l⁻¹ rifampicin, at 28°C for 3 days. Then, a single colony of transformed Agrobacterium cells was grown overnight at 28°C in liquid YEB medium, supplemented with 200 mg l⁻¹ spectinomycin and 30 mg l⁻¹ kanamycin, until an optical density (OD₆₀₀) of 1.0 was reached. Subsequently, the Agrobacterium cells were harvested and resuspended in inoculation buffer (MS salt mixture [pH 5.2]).

Lisianthus transformation

Lisianthus transformation was modified slightly a protocol described by Azuma et al. (2016). Leaf sections (5 mm×5 mm) cut from 1.5-month-old seedlings were pre-cultured on the basic medium (MS medium containing 0.5 mg l⁻¹ 6-benzylaminopurine [BAP], 0.3% (w/v) gellan gum, and 2% (w/v) sucrose at 25°C for 5 days. The leaf explants were soaked in the Agrobacterium suspension for 5 min, and then transferred to the basic medium and co-cultured in the dark at 25°C for 7 days. After co-cultivation, the explants were transferred to the selection medium (basic medium containing 50 µg ml⁻¹ hygromycin and 10 µg ml⁻¹ meropenem [Meropen, Sumitomo Dainippon Pharma Co., Osaka, Japan] for 4 weeks to induce callus formation. The calli were then transferred to the shoot regeneration medium (MS medium containing 0.8% [w/v] agar, 2% [w/v] sucrose, 0.1 mg l⁻¹ BAP, 50 µg ml⁻¹ hygromycin, and 10 µg ml⁻¹ meropenem) for 4 weeks. The regenerated shoots were separated from the calli and treated with oxybion (Bayer Crop Science, Tokyo, Japan) to induce root regeneration. After the regeneration of roots, the transgenic seedlings were transplanted in sterilized soil.

After blooming, the transgenic plants were self-pollinated, and T₁ seeds were collected. Subsequently, the T₁ seeds were sown on MS medium supplemented with 50 µg ml⁻¹ hygromycin, and then incubated for 1 month at 25°C under a 16-h light/8-h dark photoperiod. T₁ plants were acclimated and grown under the conditions described above.

Confirmation of transgenic plants

To confirm the introduction of transgenes in hygromycin-
results and stained with ethidium bromide. The copy number of T-DNA in CYP76AD1-DOD-5GT clone #1 was examined by Southern blot analysis. Briefly, genomic DNA was isolated from 5 g of young leaves of T1 seedlings of clone #1, as described previously (Nakatsuka et al. 2005). Then, 10 µg of genomic DNA samples were digested by the Hind III restriction enzyme and separated on 0.7% agarose gel. The resulting bands were transferred to Hybond-N membrane (GE Healthcare Life Science, Little Chalfont, England). The HPT ORF was detected by gene-specific labeled probes prepared using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Sigma-Aldrich, Tokyo, Japan).

Flavonoid and betalain analyses
To analyze anthocyanin and betalain pigments in T1 lines, 100 mg of petals of each T1 line were extracted with 500 µl of 1% (v/v) formic acid by incubation at 4°C for 2 h. The extracts were filtered by passage through a 0.22-µm PTFE syringe filter (Osaka Chemical, Osaka, Japan). Betalain analysis was conducted using the Agilent Infinity 1290 High-Performance Liquid Chromatography (HPLC) System (Agilent Technologies, Santa Clara, CA, USA) and Agilent 1200 Diode-Array Detector SL (Agilent Technologies) equipped with Asahipak-ODP-50 4E column (4.6 mm×250 mm, 5 µm; Showa Denko, Yokohama, Japan). Solvent A (1% [v/v] formic acid) and solvent B (80% [v/v] acetonitrile) were used for elution at 40°C at a flow rate of 1.0 ml·min⁻¹. Elution was performed with 0% B for 2 min, followed by 0–33% B for 30 min under gradient elution conditions. Betacyanins and betaxanthins were monitored based on absorbance values measured at 538 and 470 nm, respectively. Anthocyanin and flavonol contents were measured as described by Nakatsuka et al. (2019). Betalain, anthocyanin, and flavonol concentrations were evaluated based on the total peak area of each wavelength.

Petal color measurement
L* (lightness), a*, and b* (two Cartesian co-ordinates) in the fresh petals of T1 lines were measured using a color reader, CR-20 (Konica Minolta, Tokyo, Japan). Metric chroma, C*, and hue angle (h°), were calculated according to the following equations: 
C*=(a*²+b*²)⁰.⁵ and hab=−tan⁻¹(b*/a*) (Uddin et al. 2004).

Results
A total of 1,331 leaf sections of lisianthus cv. Light pink sum were inoculated with A. tumefaciens EHA101 harboring pShyg-35SpCYP76AD1-35SpDOD-35Sp5GT (CYP76AD1-DOD-5GT). Hygromycin-resistant calli were selected on shoot selection media, and 59 hygromycin-resistant shoots were regenerated from the calli. Hygromycin-resistant calli expressing CYP76AD1-DOD-5GT showed no difference in its color compared with that of the vector control (data not shown). Genomic PCR analyses confirmed the introduction of transgenes in 33 of 35 independent hygromycin-resistance shoots (data not shown). In in vitro culture, a few transgenic lines produced red leaves (Figure 3A); however, most red shoots failed to root and showed stunted growth after acclimatization (Figure 3B). Finally, one transgenic line (clone #1) successfully acclimatized and bloomed. Clone #1 produced slightly deeper pink flowers than the vector control line (Figure 3C). The leaf color of clone #1 was green, which was similar to that of the vector control line. Clone #1 was then self-pollinated to produce T1 seeds.

Some T1 seedlings derived from clone #1 showed red leaf color (Figure 3D). A total of 1,282 T1 seeds were sown on hygromycin-selection medium, of which 413 germinated (32.2%). Of the 413 seedlings, 203 (49.2%) produced red leaves. Southern blot analysis showed four band signals in CYP76AD1-DOD-5GT T1 seedlings, implying that at least four copies of T-DNA were introduced in T0 clone #1 (Supplementary Figure S1). On blooming, these T1 progenies showed five different flower colors. We selected five T1 lines, each representing a different flower color: #1-1 (wine red), #1-2 (bright pink), #1-3 (pale pink), #1-4 (pale yellow), and #1-5
The flower color of the vector control T1 line was pale pink, similar to that of line #1-4. Flower colors were stable within the same lines. The lightness index ($L^*$) values of lines #1-1 (32.2) and #1-2 (55.6) were lower than that of the vector control (78.9) (Table 1), whereas their chroma ($C^*$) values (59.1 and 46.2, respectively) were higher than that of the vector control (15.8). The $L^*$ and $C^*$ values of line #1-3 were similar to those of the vector control. The $L^*$ value of line #1-4 (85.4) was slightly higher than that of the vector control, whereas $C^*$ of line #1-5 was higher.

Next, we conducted plant pigment analyses of each line using their petals (Figure 5). In the petal extracts of lines #1-1, #1-2, and #1-5, two major betacyanin peaks were detected by HPLC (Supplementary Figure S2A), each with $\lambda_{max}$ at 535 nm (Supplementary Figure S2C). Based on their retention times and absorption spectra, these peaks were presumed to represent betanin and isobetanin. The petals of line #1-1 showed the highest betacyanin content, whereas lines #1-2 and #1-5 contained only 14.8% and 7.2%, respectively, of the betacyanins present in line #1-1 (Figure 5A). In only line #1-1, a single peak with $\lambda_{max}$ at 470 nm was detected, which implied low-level betaxanthin accumulation (Supplementary Figures S2B and S2C). The anthocyanin accumulation levels in the petals of transgenic lines were reduced significantly by 28.9–61.2% compared with those of the vector controls (Figure 5B). On the other hand, no significant difference in flavonol accumulation was detected in transgenic lines compared with vector controls, except in lines #1-4 and #1-5 (Figure 5C).

**Table 1.** Colorimetric characteristics in petals of transgenic plants.

| Line       | $L^*$  | $a^*$  | $b^*$ | $C^*$  | $\Delta E^*$ |
|------------|--------|--------|--------|--------|--------------|
| Vector control | 78.9   | 15.7   | 2.0    | 15.8   | 7.1          |
| CYP76AD1-DOD-5GT | #1-1   | 32.2   | 57.9   | -12.0  | 59.1         | -11.7        |
|             | #1-2   | 55.6   | 45.3   | -9.3   | 46.2         | -11.6        |
|             | #1-3   | 77.5   | 19.8   | 0.3    | 19.8         | 0.8          |
|             | #1-4   | 85.4   | 6.8    | 18.2   | 19.4         | 69.6         |
|             | #1-5   | 67.3   | 33.0   | 3.4    | 33.2         | 5.9          |
The expression levels of transgenes in the petals of each transgenic T₁ line were investigated by semi-quantitative RT-PCR (Figure 6). The CYP76AD1 showed expression levels in all transgenic lines. DOD showed the highest expression in line #1-1, followed by lines #1-2, #1-5, and #1-3. 5GT was expressed to higher levels in lines #1-1, #1-2, and #1-5 than in line #1-3. In the petals of line #1-4, transcripts of both DOD and 5GT were almost undetectable. The expression profiles of the three transgenes observed in the petals were reproduced in the leaves of each clone (Supplementary Figure S3). Additionally, expression levels of endogenous anthocyanin biosynthetic genes, CHS and DFR, showed no differences between CYP76AD1-DOD-5GT transgenic plants and vector control plants (Figure 6).

**Discussion**

In this study, we successfully modified flower color in lisianthus by accumulating betalain pigments through genetic engineering. The heterologous-expression of CYP76AD₁, DOD, and 5GT led to the de novo production of betacyanin pigments in transgenic lisianthus plants (Figure 3A). Most transgenic shoots with red leaves failed to grow after acclimatization (Figure 3B), whereas most vector control shoots were induced rooting and grown normally. When we attempted to transform tobacco (cv. SR-1) with CYP76AD₁-DOD-5GT, the transgenic plants did not exhibit delayed or stunted growth (data not shown). In a previous study, transgenic potato, eggplant, tomato, tobacco, and petunia plants expressing CYP76AD₁, DOD, and 5GT showed red tissues containing betacyanin pigments (Polturak et al. 2017). Although
these transgenes were also under the control of the CaMV35S promoter, the transgenic plants showed normal growth (Polturak et al. 2017). To our knowledge, there is no previous report on betalain accumulation affecting plant growth. In case of anthocyanins instead of betalains, transgenic Arabidopsis, tomato, petunia, and apple lines expressing the maize Lc, which encodes an anthocyanin biosynthesis regulator, showed enhanced ectopic anthocyanin accumulation but failed to regenerate shoots and roots (Bradley et al. 1998; Li et al. 2007; Quattrocchio et al. 1993). Thus, as the abundant accumulation of plant pigments might cause growth inhibition in some plant species, the biological function of betalains might be similar to that of anthocyanins. Another reason that may explain growth inhibition is the accumulation of tyrosine. Tyrosine, which is the primary precursor of betalain, is synthesized by arogenate dehydrogenase (ADH). ADH enzymes are strongly feedback inhibited by tyrosine, and control carbon flow between two competing tyrosine and phenylalanine pathways (Lopez-Nieves et al. 2018). ADHα derived from betalain-producing plant species exhibits relaxed sensitivity to tyrosine. Therefore, tyrosine deficiency induced by betalain biosynthesis could be another reason why transgenic lisianthus plants exhibited growth inhibition, although further investigation is necessary.

T1 progenies of CYP76AD1-DOD-5GT (117–148 days to anthesis from planting) also showed a slower growth rate than those of the vector control (104 days) but were not stunted during cultivation (Figures 3D, 4). Betalain-accumulated T1 progenies might have grown because they have normal root. The T1 progeny derived from clone #1 showed five flower colors: wine red (#1-1), bright pink (#1-2), pale pink (#1-3), pale yellow (#1-4), and salmon pink (#1-5) (Figure 4). Although cv. Light pink sum is an F1 cultivar, the T1 progeny of the vector control produced only pale pink flowers (data not shown). Therefore, the different flower colors obtained in the T1 progeny of clone #1 are resulted likely from the expression of the three transgenes, CYP76AD1, DOD and 5GT. The flower color intensities of lines #1-1 and #1-2 were more vivid red–violet than that of T0 clone #1 (Figures 3C, 4).

The difference in flower color among the T1 progenies depended on the betalain and anthocyanin accumulation levels (Figure 5). Lines #1-1, #1-2, and #1-5 accumulated betacyanins in their petals, whereas lines #1-3 and #1-4 did not (Figure 5A). In only line #1-1, low-level of betaxanthin also detected in its petals (Supplementary Figure S2). These transgenic lines showed strong expression levels of all three transgenes, which were correlated well with the betacyanin accumulation levels (Figures 5, 6). The expression levels of DOD and 5GT were low in lines #1-3 and #1-4, which did not produce betacyanins. These data suggest that synchronous and strong expression levels of the three betalain biosynthetic genes, CYP76AD1, DOD, and 5GT, are necessary to produce betalain in transgenic lisianthus. Southern blot analysis revealed the existence of four T-DNA copies in T0 clone #1 (Supplementary Figure S1). We speculated that the difference in flower color among T1 lines might be caused by the difference in allele numbers (homozygous or heterozygous) and/or the DNA methylation status of T-DNA. However, our results clearly indicated variations in CYP76AD1 expression levels among T1 lines. Further studies are necessary to elucidate the molecular basis of different flower colors among T1 lines by determining the exact expression levels of transgene during flower development via quantitative RT-PCR and by evaluating T-DNA allele numbers. Anthocyanins and betalains generally exhibit mutually exclusive distribution in plants (Timoneda et al. 2019). This study revealed that betalain and anthocyanin pigments can co-exist within a plant species using transgenic engineering. Interestingly, anthocyanin accumulation was suppressed in all transgenic lines, especially betalain-accumulated lines #1-1, #1-3, and #1-5 were reduced by 28.9–61.2% compared with vector controls (Figure 5B). However, the expression levels of endogenous anthocyanin biosynthetic genes, CHS and DFR, did not show major differences among the transgenic lines (Figure 6). Although we did not analyze the expression level of the FLS, flavonol contents were reduced significantly in lines #1-4 and #1-5 compared with the control (Figure 5C). Not only the expression levels of other anthocyanin biosynthetic genes but also enzymatic activity, transport, and accumulation might be affected by de novo betalain production in transgenic lisianthus. Further studies are necessary to reveal the competition between the accumulation of betalains and anthocyanins in plants. CYP76AD1-DOD-5GT lisianthus plant produced in this study might useful to reveal mystery of exclusive distribution between betalains and anthocyanins.

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Disclosure of potential conflict of interests

There are no conflicts of interest to be declared.

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