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A basic helix-loop-helix transcription factor DvIVS determines flower color intensity in cyanic dahlia cultivars

Sho Ohno1* • Ayumi Deguchi1 • Munetaka Hosokawa1* • Fumi Tatsuzawa2 • Motoaki Doi1

1Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
2Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

*Corresponding author: Sho Ohno
Laboratory of Vegetable and Ornamental Horticulture, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
Telephone: +81-75-753-6048, Fax: +81-75-753-6068
E-mail: ono.sho.47n@st.kyoto-u.ac.jp

*Corresponding author: Munetaka Hosokawa
Laboratory of Vegetable and Ornamental Horticulture, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
Telephone: +81-75-753-6048, Fax: +81-75-753-6068
E-mail: mune@kais.kyoto-u.ac.jp

Ayumi Deguchi E-mail: a.deguchi@ax3.ecs.kyoto-u.ac.jp
Fumi Tatsuzawa E-mail: fumi@iwate-u.ac.jp
Motoaki Doi E-mail: dmo.toaki@kais.kyoto-u.ac.jp
Abstract
The study was aimed to identify the factors that regulate the intensity of flower color in cyanic dahlia (*Dahlia variabilis*), using fifteen cultivars with different color intensities in their petals. The cultivars were classified into three groups based on their flavonoid composition: ivory white cultivars with flavones; purple and pink cultivars with flavones and anthocyanins; and red cultivars with flavones, anthocyanins, and chalcones. Among the purple, pink, and ivory white cultivars, an inverse relationship was detected between lightness, which was used as an indicator for color intensity and anthocyanin content. A positive correlation was detected between anthocyanin contents and the expression of some structural genes in the anthocyanin synthesis pathway that are regulated by *DvIVS*, a basic helix-loop-helix transcription factor. A positive correlation between anthocyanin content and expression of *DvIVS* was also found. The promoter region of *DvIVS* was classified into three types, with cultivars carrying Type 1 promoter exhibited deep coloring, those carrying Type 2 and/or Type 3 exhibited pale coloring, and those carrying Type 1 and Type 2 and/or Type 3 exhibited medium coloring. The transcripts of the genes from these promoters encoded full length predicted proteins. These results suggested that the genotype of the promoter region in *DvIVS* is one of the key factors determining the flower color intensity.

Keywords
Anthocyanin, bHLH, Correlation analysis, Dahlia, Flower color intensity

Abbreviations
ANS Anthocyanidin synthase
bHLH Basic helix-loop-helix
CHI Chalcone isomerase
CHS Chalcone synthase
DFR Dihydroflavonol 4-reductase
F3H Flavanone 3-hydroxylase
FNS Flavone synthase
HPLC High performance liquid chromatography
Introduction

Color is one of the most important characters of ornamental traits in flowers, and breeders strive to develop new exotic colors each day. Flower color results due to accumulation of secondary metabolites such as flavonoids including anthocyanins, carotenoids, and/or betalains. Generally, higher amounts of pigments deepen color intensity (Grotewold, 2006, Tanaka et al., 2008). Inversely, lower pigment amounts in pigmented organs lead to paler coloring in carnation (Dianthus caryophyllus), eustoma (Eustoma grandiflorum), delphinium (Delphinium), and pelargonium (Pelargonium × domesticum) (Mato et al., 2001, Uddin et al., 2004, Hashimoto et al., 2000, Fujioka et al., 1991). In some cases, other factors such as cell shape and vacuolar pH strongly influence the flower color intensity (Mol et al., 1998).

Garden dahlia (Dahlia variabilis) is one of the most popular ornamental plants in the world. In particular, dahlia has more abundant flower color variations than other floricultural species, with red, purple, pink, ivory white, and black cultivars resulting due to accumulation of anthocyanin, flavone, and/or butein derivatives (Takeda et al., 1986, Nordström and Swain, 1953, Nordström and Swain, 1956, Nordström and Swain, 1958, Bate-Smith and Swain, 1953, Bate-Smith et al., 1955, Harborne et al., 1990, Price, 1939, Deguchi et al., 2013). These flavonoids are synthesized in the flavonoid biosynthetic pathway, one of the most profusely studied plant secondary metabolite pathways. Flavonoids are initially synthesized in the phenylpropanoid synthesis pathway, and subsequently anthocyanins are synthesized in the anthocyanin synthesis pathway. Three molecules of malonyl-CoA and one molecule of 4-coumaroyl CoA are condensed by chalcone synthase (CHS), followed by chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) to synthesize anthocyanidin (Tanaka et al., 2008). Transcription of some of these genes is promoted by a transcription complex composed of basic helix-loop-helix (bHLH), R2R3-MYB, and WD-repeat proteins (Koes et al., 2005, Hichri et al., 2011). The large variety of flower color in dahlias is presumably due to its genetic background. Garden dahlia is thought to be an autoallooctoploid with 2n = 8x = 64 (Gatt et al., 1998), and this complicated genetic background has led to the development of more than 50,000 cultivars in the past 100 years (McClaren, 2009). Highly polyploid species should offer more number of allelic combination and loci than diploid species, and correspondingly more complicated mechanisms are expected in the regulation of a given trait such as anthocyanin synthesis.

In a previous study (Ohno et al., 2011a), we demonstrated that a bHLH transcription factor DvIVS, belonging to the subgroup of petunia anthocyanin1 (Spelt et al., 2000), Arabidopsis thaliana TT8 (Nesi et al., 2000), and common morning glory (Ipomoea purpurea) IpIVS (Park et al., 2007), is associated with anthocyanin synthesis via regulation of DvCHS1, DvF3H, DvDFR, and DvANS, but not DvCHS2 and DvCHI (Fig. 1). In another previous study (Ohno et al., 2011b), due to absence of expression of DvIVS in commercial white (ivory white) cultivars derivatives of anthocyanidin were not produced. These results indicate that non-expression of both DvIVS and butein synthesis pathway genes
results in ivory white flowers, whereas expression of butein synthetic genes without the expression of DvIVS results in yellow flowers. In contrast, the expression of DvIVS without the expression of butein synthetic genes results in purple flowers, whereas expression of both DvIVS and butein synthetic genes results in red flowers. But there are paler color cultivars such as pink and orange cultivars in dahlia, and the mechanisms determining color intensity are unknown. We accordingly developed and tested a hypothesis that expression level of DvIVS determines the amount of anthocyanin and thereby flower color intensity. Because it is also likely that there are multiple DvIVS alleles in dahlia owing to its high polyploidy, we also investigated the involvement of a specific DvIVS genotype in the regulation of flower color intensity.

**Materials and methods**

**Plant materials**

We chose fifteen cultivars according to flower color: purple cultivars ‘Super Girl’, ‘Yukino’, ‘Cupid’, ‘Evelyn Rumbold’ and ‘Atom’; pink cultivars: ‘Magokoro’, ‘Jynn-ai’ and ‘Saffron’; ivory white cultivars ‘Gitt’s Attention’, ‘Zannsetsu’, ‘Hakuba’ and ‘Hakuyo’; and red cultivars ‘Agitato’, ‘Nekkyu’ and ‘Red Velvet’ (Fig. 2a-o). Some cultivars were purchased from Akita International Dahlia Park (Akita, Japan). All cultivars were grown in the experimental farm of Kyoto University (Kyoto, Japan) and their petals were used for this study. For inverse PCR analysis, we used genomic DNA extracted from a red-white bicolor cultivar ‘Yuino’ (Ohno et al. 2011b).

**Color analysis**

Color components of the CIE L*a*b* co-ordinate were measured with the purpose of describing petal color differences of statistical significance. Color analyses were carried out following the Commission Internationale de l'Eclairage system. L* indicates Lightness (black: 0 to white: 100). Positive a* values indicate redness and negative a* values indicate greenness. Positive b* values indicate yellowness and negative b* values blueness. Chroma (c*), purity or saturation of the color, was calculated as follows: 

\[ c^* = (a^*2 + b^*2)^{1/2} \]

The L*, a*, and b* were measured with a hand held spectrophotometer (NR-3000, Nippon Denshoku Industries Co., Ltd., Tokyo, Japan). Three areas of the adaxial surface were subjected to color measurement. A mean score of the three replicate petals from three different flowers was calculated.
High performance liquid chromatography (HPLC) analysis

To determine the composition of flavonoids and anthocyanidins, HPLC analysis was performed according to Ohno et al. (2011a). In summary, petals were homogenized with acetic acid: methanol: water (1: 4: 5 v/v) solution for pigment extraction. The extracts were dried and re-dissolved in 2 mL of 20% hydrochloric acid. This solution was heated to evaporate the solvent, and 500 µL of 20% hydrochloric acid was added to measure crude aglycons. HPLC analysis was performed using an LC10A system (Shimadzu, Kyoto, Japan) with a C18 column (Nihon Waters K.K., Tokyo, Japan) maintained at 40°C and a photodiode array detector. The detection wavelength was 350 nm for flavone and chalcone aglycones and 530 nm for anthocyanin aglycones.

Anthocyanin measurement

For anthocyanin quantification, 100 – 200 mg of fresh petals were homogenized in liquid nitrogen and 1 mL of extraction buffer (acetic acid: methanol: water = 1: 4: 5 v/v) was added. The extracted samples were centrifuged for 2 min at 20,600 × g and the supernatant was collected. The supernatant was diluted 10 or 100 fold with the extraction buffer and the absorption at 520 nm was measured using a double beam spectrophotometer (U-2000A, Hitachi, Tokyo, Japan). The anthocyanin amount per 100 mg of fresh petals was recorded. A standard curve was prepared using cyanidin chloride (Polyphenols, Norway). The assay was performed with three petals from three independent inflorescences.

Other characteristics of petals

The pH of the petals was measured using previously described methods (Quattrocchio et al., 2006). Petals (200 mg) were ground in 6 mL of distilled water. The pH was directly measured with a pH meter within 1 min. A mean of the three values was used for further data analysis.

To determine whether pigments were accumulating only in epidermal cells, fresh sections of ray florets were observed using a VHX-100 digital microscope (KEYENCE, Osaka, Japan). Epidermis cell structures were examined in resin sections prepared as follows. Petals were fixed in an FAA (ethanol: water: formalin: acetic acid, 12: 6: 1: 1 v/v) solution and cut into 5 mm squares. Samples were dehydrated in a graded ethanol series, and subsequently exchanged for Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) by immersing the samples in liquid resin for more than 3 h. Samples were solidified in the resin according to the manufacturer’s protocol. The embedded samples were cut into 5 µm sections using a motorized rotary microtome (RM2155, Leica, Wetzlar, Germany). Sections were stained with a 0.05% toluidine blue solution for 30 min and washed with water for 5 min. The dyed sections were observed and photographed with a VHX-100 digital microscope (KEYENCE).
Real-time RT-PCR

Total RNA was extracted with Sepasol®-RNA I Super G (Nacalai Tesque, Kyoto, Japan), purified with High-Salt Solution for Precipitation (Takara Bio Inc., Ohtsu, Japan), and reverse transcribed with ReverTra Ace (TOYOBO, Osaka, Japan), and 2 µL of 50-fold diluted RT product was used as template for real-time RT-PCR. Real-time RT-PCR was performed with SYBR® Premix Ex Taq™ II (Takara Bio Inc.) according to the manufacturer’s instructions using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used are shown in Supplementary Table S1. The PCR program was set at 95°C for 10 s, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and subsequent dissociation steps. Three technical replications for two biological replications were performed and actin was used for an internal standard.

Correlation analysis

Correlations between L* and anthocyanin amount, L* and vertical-horizontal ratio of epidermal cell shape, L* and petal pH, anthocyanin content and anthocyanin synthesis pathway genes, and anthocyanin synthesis genes and DvIVS expressions were calculated. All correlation analysis was performed by linear approximation method using Microsoft Excel.

Isolation of DvIVS promoter region

The promoter region of DvIVS was isolated by inverse PCR. Genomic DNA of ‘Yuino’ (Ohno et al. 2011b) petals was isolated using a modified cetyltrimethylammonium bromide method (Murray and Thompson, 1980) and purified with MagExtractor™-Plant Genome- (TOYOBO). One microgram of genomic DNA was digested with Hind III (TOYOBO) and enzymes were removed with phenol/chloroform/ isoamyl alcohol (25: 24: 1) (Nacalai Tesque). After ethanol precipitation, 200 ng of digested DNA, 350 U of T4 DNA ligase (Takara Bio Inc.), and 10 × buffer were mixed and water was added to 20 µL. The mixture was incubated at 16°C overnight and PCR was performed with LA Taq (Takara Bio Inc.) using 1 µL of ligation product as a template in a 10 µL volume. The PCR program was set at 94°C for 1 min, followed by 35 cycles of 98°C for 10 s, 55°C for 10 s, and 68°C for 15 min. Primers used in this PCR were IVS-113R and IVS-G1163F (Supplementary Table S2) designed for our reported sequence (AB601010). PCR products were cloned into pTAC-1 vectors using Dyna-Express TA PCR Cloning Kit (BioDynamics Laboratory Inc., Tokyo, Japan) and all sequencing was performed using a BigDye ® Terminator v 3.1 Cycle Sequencing Kit and a 3100 Genetic Analyzer (Applied Biosystems).
Genotyping of *DvIVS* promoter and cDNA sequences

For genotyping of *DvIVS* promoter region, primers were designed according to isolated promoter region sequence as shown in Supplementary Table S3 and PCR was performed with Blend Taq polymerase (TOYOBO) through the following steps and subsequently sequenced: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 4 min.

The *DvIVS* cDNAs in ‘Super Girl’, ‘Yukino’, ‘Cupid’, ‘Evelyn Rumbold’, ‘Atom’, ‘Magokoro’, ‘Jyun-ai’ and ‘Saffron’ were amplified with Blend Taq polymerase (TOYOBO) using IVS Full-F and IVS Full-R primers. The PCR products were cloned and sequenced as described above, using primers shown in Supplementary Table S2 designed for our reported sequence (AB601010). Subsequently, primers were designed to detect the specific transcript (Supplementary Table S4) and were amplified by RT-PCR with KOD FX polymerase (TOYOBO) through the following steps: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 68°C for 2 min, and a final extension at 68°C for 5 min.

To amplify the combination of promoter and transcribed RNA types, PCR was performed using newly designed primers shown in Supplementary Table S4 and S5 with Blend Taq polymerase (TOYOBO) through the following steps: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. Twelve primer combinations (3 promoter type × 4 mRNA types) were analyzed.

Results

Flavonoid compositions and color differences

As difference among cultivars with respect to the modification of anthocyanidins and flavones was not observed (data not shown), the hydrolyzed aglycones were analyzed. In ivory white cultivars, only flavones, apigenin, and luteolin were detected, whereas in pink and purple cultivars, cyanidin, and pelargonidin were detected in addition to flavones (Table 1). In red cultivars, flavones, anthocyanidins, and chalcones, isoliquiritigenin and butein were detected (Table 1). Sulfuretin (aurone) was undetected or detected only at trace levels in all cultivars (Table 1). To simplify the experiments, only purple, pink, and ivory white cultivars were used for further the study.

Because deeper color generally shows lower L* value, L* value was used as an indicator of flower color intensity. When the color characteristics of 12 cultivars were plotted with c* on the X axis...
and \( L^* \) on the \( Y \) axis, they could be classified into three groups corresponding to their appearances (Fig. 3). Ivory white cultivars had the highest \( L^* \) and the lowest \( c^* \) value. In contrast, purple cultivars had the lowest \( L^* \) and the highest \( c^* \) value. Pink cultivars had intermediate values for both the indices.

Anthocyanin contents and pH measurement

The anthocyanin contents of the 12 cultivars are shown in Fig. 4a. Purple cultivars contained the highest, with ‘Super Girl’ and ‘Yukino’ containing exceedingly high amounts, 0.7–1.0 mg anthocyanin per 100 mg petals. Other purple cultivars (‘Cupid’, ‘Evelyn Rumbold’ and ‘Atom’) contained 0.3–0.4 mg per 100 mg petals. We accordingly designated the former as deep purple cultivars and the latter as purple cultivars. Pink cultivars had lower anthocyanin amounts than purple cultivars, with <0.1 mg. In ivory white cultivars, no anthocyanin was detected, which was consistent with the HPLC results (Table 1). An inverse relationship between \( L^* \) and anthocyanin content was observed among eight cyanic (deep purple, purple, and pink) cultivars (Fig. 4b), suggesting that anthocyanin content was the key factor determining flower color intensity (\( L^* \)) in these cultivars.

The petal pH of the cultivars examined was 4.9–5.8 (Supplementary Table S6). No significant correlation was detected between \( L^* \) and petal pH (data not shown), suggesting that petal pH does not contribute to \( L^* \).

Observation of petal sections

Observation of sections of fresh petals showed that anthocyanin accumulated only in epidermis (Supplementary Fig. S1a-d). No characteristic morphology was observed in resin sections of the eight cultivars (Supplementary Fig. S1e-h). To confirm the correlation between \( L^* \) and the vertical–horizontal ratio was analyzed, but no significant correlation was detected (\( r = -0.50 \): Supplementary Fig. S1i).

Real-time RT-PCR analysis

In a previous study (Ohno et al., 2011a), we isolated anthocyanin synthesis structural genes and bHLH transcription factor \( DvIVS \), which regulates the expression of \( DvCHS1, DvF3H, DvDFR, \) and \( DvANS \). In the real-time RT-PCR analysis of \( DvCHS1, DvF3H, DvDFR, \) and \( DvANS \), comparatively high expression levels were measured in ‘Super Girl’ and ‘Yukino’ and little or no expression was detected in ivory white cultivars (Fig. 5). The same expression patterns were detected for \( DvIVS \) (Fig. 5). In contrast, high expressions were detected in ivory white cultivars for \( DvCHS2 \) and \( DvCHI \) (Fig. 5). Other transcription factors, \( DvMYB1, DvMYB2, DvR3MYB, DvDEL \) and \( DvWDR1 \) showed different expression patterns from \( DvCHS1, DvF3H, DvDFR, DvANS \) and \( DvIVS \) (Supplementary Fig. S2).
We performed correlation analysis to investigate the relationships among anthocyanin contents, anthocyanin synthesis structural gene expression, and DvIVS expression. The $r$ values (correlation coefficient) between anthocyanin content and DvCHS1, DvCHS2, DvCHI, DvF3H, DvDFR, and DvANS expression were 0.97, 0.09, 0.15, 0.87, 0.69, and 0.90, respectively (Fig. 6a-f). The $r$ values between DvIVS and DvCHS1, DvCHS2, DvCHI, DvF3H, DvDFR, and DvANS expression were 0.99, 0.10, 0.22, 0.96, 0.81, and 0.97, respectively (Fig. 7a-f). The $r$ value between anthocyanin contents and DvIVS expression was 0.96 (Fig. 6g), showing that anthocyanin content and the expression of DvIVS were strongly and positively correlated. In contrast, the $r$ values between anthocyanin content and DvMYB1, DvMYB2, DvR3MYB, DvDEL, and DvWDR1 expression were 0.01, 0.41, 0.41, 0.32, and 0.39, respectively (data not shown).

Length polymorphisms in DvIVS promoter region

Because the expression levels of a gene are usually affected by its promoter region, we isolated the promoter region of DvIVS. Fragments of 253 bp upstream of the DvIVS mRNA transcription start site were isolated by inverse PCR using ‘Yuino’, and this obtained promoter sequence was subsequently named Type 2. When we designed primers and PCR was performed using cyanic cultivars, there were three length polymorphisms were detected. These three fragments were sequenced and named Type 1–3.

Type 1 promoter was detected in the deep purple and purple cultivars (‘Super Girl’, ‘Yukino’, ‘Cupid’, ‘Evelyn Rumbold’ and ‘Atom’) by using IVS-2F and IVS-113R primers (Fig. 8a). Type 2 promoter was detected in ‘Cupid’, ‘Evelyn Rumbold’, ‘Atom’, ‘Jyunn-ai’, ‘Saffron’ and ‘Hakuyo’ by using IVS-3F and IVS-113R primers (Fig. 8a). Type 3 promoter was detected in ‘Cupid’, ‘Magokoro’, and ‘Saffron’ by using IVS-4F and IVS-113R primers (Fig. 8a). Thus deep purple cultivars carried only Type 1 promoters, purple cultivars carried Type 1 and Type 2 or all three promoters, pink cultivars carried Type 2 and/or Type 3 promoter(s), and ivory white cultivars except for ‘Hakuyo’ carried none of the promoter types (Fig. 8a). Type 2 promoter had a 125 bp insertion just behind the IVS-2F primer sequence, and Type 3 had another 103 bp insertion just behind IVS-3F primer sequence instead of 40 bp Type 2 specific fragment (Fig. 8b). In the putative TATA-box region, Type 1 had TTAAGTAG, while Type 2 and Type 3 had TTAAATAG (Fig. 8c).

Analysis of DvIVS transcripts and combination with promoter region

To analyze the relationship between polymorphisms of DvIVS promoter region and DvIVS mRNA expression levels, DvIVS cDNA from all cyanic cultivars were sequenced. Four different DvIVS cDNA sequences were isolated and named mRNA-1 to mRNA-4 (AB787557–AB787560) based on the differences at amino acid positions 235–295 of the putative protein (Supplementary Fig. S3). All these
mRNAs might encode complete putative proteins.

To determine the expressing mRNA types in cyanic cultivars, RT-PCR was performed using type-specific primers for each mRNA (Supplementary Table S4). mRNA-1 was detected in the deep purple and purple cultivars (‘Super Girl’, ‘Yukino’, ‘Cupid’, ‘Evelyn Rumbold’ and ‘Atom’); mRNA-2 in ‘Yukino’, ‘Cupid’ and ‘Jyunn-ai’; mRNA-3 in ‘Magokoro’ and ‘Saffron’; and mRNA-4 in ‘Evelyn Rumbold’ and ‘Saffron’ (Table 2).

Further, promoter–mRNA combinations were investigated to confirm that Type 1 promoter actually encodes a functional protein using specific primers (Supplementary Table S5). Consequently, the combinations of Type 1 promoter–mRNA-1, Type 2 promoter–mRNA-2, Type 3 promoter–mRNA-3, and Type 2 promoter–mRNA-4 were inferred (Table 2). Type 1 promoter–mRNA-1 combination was detected in the deep purple and purple cultivars (‘Super Girl’, ‘Yukino’, ‘Cupid’, ‘Evelyn Rumbold’ and ‘Atom’); Type 2 promoter–mRNA-2 in ‘Cupid’ and ‘Jyunn-ai’; Type 3 promoter–mRNA-3 in ‘Magokoro’ and ‘Saffron’; and Type 2 promoter–mRNA-4 in ‘Evelyn Rumbold’ and ‘Saffron’ (Table 2). The coding region associated with ‘Atom’ and ‘Hakuyo’ Type 2 was not identified, but no full length DvIVS transcript for this promoter was detected, it was suggested this promoter might be non-functional. Although, the promoter region associated with ‘Yukino’ mRNA-2 and the coding region associated with ‘Cupid’ Type 3 promoter, were neither identified, all deeply colored (deep purple and purple) cultivars retained Type 1 promoter–mRNA-1 combination, suggesting this allele is important for high anthocyanin accumulation.

Discussion

Anthocyanin contents determine flower color intensities in cyanic dahlia cultivars

In this study, the factors determining the flower color intensity of cyanic dahlia cultivars were analyzed. Usually flower color is determined by pigment composition and amount. We could classify fifteen cultivars into three groups by flavonoid composition: cultivars with flavones, cultivars with flavones and anthocyanins, and cultivars with flavones, anthocyanins, and butein (Table 1). All commercial red cultivars belonged to the last group, indicating that the purple anthocyanin pigments and the yellow pigment butein confer red flower color, and were excluded from further analysis. The twelve cultivars containing anthocyanins and/or flavones were used for further studies.

Although no difference in flavonoid composition between deep purple, purple, and pink cultivars was observed, their L* values as an indicator of flower color intensity were different (Fig. 3, Supplementary Table S6). Anthocyanin contents and L* values of the twelve cultivars showed an inverse relationship (Fig. 4b), suggesting that the anthocyanin contents determined flower color intensities.
Pigments accumulated in epidermal cells of petals in all cultivars (Supplementary Fig. S1a-d). In the snapdragon (*Antirrhinum majus* mixta) mutant, cell shape is associated with flower intensity (Noda et al., 1994). From a comparative observation of epidermal cell shape, no relationship was detected between flower color intensity and cell shape (Supplementary Fig. S1i), indicating that cell shape is not involved in flower color intensity in cyanic dahlias. In Japanese morning glory (*Ipomoea nil*), an increase in vacuolar pH is correlated with bluish flower display (Yoshida et al., 1995). In our study, no significant correlation between L* value and petal pH was detected (data not shown) suggesting that petal pH contributes little to flower color intensity. From these results, we concluded that the quantity of total anthocyanins was the principal factor determining flower color intensity in cyanic dahlia cultivars.

Quantitative levels of *DvIVS* transcripts correlate flower color intensity by regulating anthocyanin content

In general, anthocyanin synthesis is regulated by bHLH, MYB, and WDR transcription factors (Koes et al., 2005). Overexpression of one of these transcription factors induced high accumulation of anthocyanin in the flower (Laitinen et al., 2008, Pattanaik et al., 2010, Bai et al., 2011). In petunia, common morning glory and tobacco, bHLH transcription factors have been shown to be one of key factors for floral anthocyanin biosynthesis (Spelt et al., 2000, Park et al., 2007, Bai et al., 2011). In dahlia, we reported a bHLH transcription factor *DvIVS* regulates anthocyanin synthesis (Ohno et al., 2011a), and thus it was assumed that the expression levels of *DvIVS* determined flower color intensity in dahlia.

In the present study, expressions of *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS* were strongly co-ordinated with both anthocyanin content and the expression of *DvIVS* (Fig. 6a-f, 7a-f). A positive correlation between expression levels of *DvIVS* and anthocyanin content was detected (Fig. 6g). Similarly, in tepals of Asiatic hybrid lilies a positive correlation between anthocyanin content and *LhMYB12* was found (Yamagishi et al., 2012). In addition, we analyzed expression levels of *DvMYB1* (AB601003), *DvMYB2* (AB601004), *DvR3MYB* (AB621921), *DvDEL* (AB601006), and *DvWDR1* (AB601007) which are highly homologous to anthocyanin-regulating transcription factors (Supplemental Fig. S2), but no significant correlation was detected between the expression levels of these transcripts and anthocyanin synthesis gene expression or anthocyanin content. We accordingly suggest that *DvIVS* determines the anthocyanin content via regulation of structural genes, thereby regulating intensity of flower color.

The genotype of the *DvIVS* promoter region corresponds to the expression levels of *DvIVS*

We could classify the twelve cultivars into four groups: deep purple cultivars with high anthocyanin content (‘Super Girl’ and ‘Yukino’), purple cultivars with moderate anthocyanin content (‘Cupid’, ‘Evelyn Rumbold’ and ‘Atom’), pink cultivars with low anthocyanin content (‘Magokoro’, ‘Jyunn-ai’ and ‘Saffron’), and ivory white cultivars without anthocyanin (‘Gitt’s Attention’, ‘Zannsetsu’, ‘Hakuba’
and ‘Hakuyo’) (Fig. 4a). The observation of higher expression levels of \( DvIVS \) in deep purple and purple cultivars carrying Type 1 promoter than in the other cultivars suggested that the genotype of promoter region of \( DvIVS \) is an important factor in flower color intensity. There was only one allele with Type 1 promoter (mRNA-1) and Type 3 promoter (mRNA-3), whereas there were at least three alleles with Type 2 promoter (mRNA-2, mRNA-4, and an unidentified nonfunctional allele). However, all \( DvIVS \) transcripts except for the unidentified one encoded a full-length predicted protein and retained the same bHLH domain (Supplementary Fig. S3). This finding suggests that high accumulation of anthocyanin resulted from the total quantity of \( DvIVS \) transcripts rather than from protein activity encoded by mRNA-1 and that the difference in expression levels of \( DvIVS \) was due to the difference in the promoter activity. If that inference is true, Type 1 promoter has a stronger activity than Type 2 or Type 3 promoters. Two hypotheses may account for the difference in promoter activity. First, a single nucleotide polymorphism was found in the putative TATA box of the promoter, which is believed to be important for the transcriptional activity. Type 1 promoter had TTAAGTAG whereas Type 2 and Type 3 promoters had TTAAATAG upstream of the transcription initiation site (Fig. 8c). This G to A mutation is the probable cause of low expression. Second, the promoter region itself accounts for the differential expressions. The genomic structure of Type 2 and Type 3 promoter had insertions with respect to Type 1 promoter (Fig. 8b). The upstream region near the transcription initiation site is very important for its expression, thus the insertion lowers the expression of \( DvIVS \).

There appeared to be at least two different non-functional alleles. The first allele was detected in ‘Hakuyo,’ which had a Type 2 promoter. ‘Hakuyo’ did not express full length transcripts (Ohno et al., 2011b), but expressed the 5’ untranslated region of \( DvIVS \) (data not shown). This observation indicated that some genomic rearrangement(s) in the coding region led this allele to non-functional. The second was an unidentified allele which other ivory white cultivars would have. Although, we cannot exclude the possibility that alleles act as a negative regulator such as AtMYBL2 (Dubos et al., 2008, Matsui et al., 2008) and CPC (Zhu et al., 2009), however, a part of coding region of \( DvIVS \) were detected from these ivory white cultivars’ genome (data not shown), suggesting some genomic rearrangement(s), perhaps in the promoter region, might lead this allele to non-functional. Thereby, further study of the genomic structure of ivory white cultivars will be required to clear the non-functional \( DvIVS \) alleles.

A functional allele is important for berry color in grape species (\( Vitis \times labruscana \) and \( V. vinifera \)); that is, berry skin color is determined by the number of functional haplotype in MYB A genes (Kobayashi et al., 2002, Kobayashi et al., 2005, Kobayashi, 2009, Azuma et al., 2011). Although we could not identify the promoter region of mRNA-2 in ‘Yukino’, the observation that deep purple cultivars carried only the stronger \( DvIVS \) promoter, whereas purple cultivars carried a weaker promoter in addition to the stronger promoter, indicated that the functional combination of the \( DvIVS \) promoter region determines flower color intensity in dahlia. To confirm this theory, the number of alleles should be measured using a digital PCR. But at least, Type 1 promoter explains deep coloring, given that 23 of 55
tested cultivars or seedling lines carried Type 1 promoter and 22 of 23 cultivars showed deep coloring (purple, red or black) (Ohno et al., unpublished data).

Genetic background for flower color of dahlia

Dahlias have one of the largest numbers of cultivars of any cultivated species. Not only flower shape and size, but color variation is very large, with combinations of these factors distinguishing thousands of cultivars. This rich variation may be due to its highly polyploidy genetic background. Genetic redundancy is one of the advantages of polyploid species (Comai, 2005); however, from a breeding perspective it may interfere with the rapid development of new cultivars with desirable traits.

In the 1920s and 30s, Lawrence and colleagues proposed four elements of dahlia flower color inheritance; A: pale anthocyanin, B: deep anthocyanin, I: ivory flavone, and Y: yellow flavone (butein) (Lawrence, 1929, Lawrence and Scott-Honcrieff, 1935). In their reports, B (deep anthocyanin) was dominant to A (pale anthocyanin). Applying these factors to cultivars used in this experiment, pink cultivars might carry only A and purple cultivars might carry B. Thus, it is expected that the weakly functioning DvIVS Type 2 and Type 3 might correspond to factor A and the strongly functioning DvIVS Type 1 might correspond to B, and that I might correspond to the non-functional DvIVS allele carried by ivory white cultivars. Yellow or red cultivars usually contain buteins as a yellow pigment, and cultivars without buteins contain neither butein nor its precursor, isoliquiritigenin. We have shown that anthocyanin synthesis and butein synthesis are mutually independent in dahlia (Ohno et al., 2011a), thus the Y element might correspond to the chalcone reductase gene or its transcription regulation factor.

In this study, it is suggested that more than six DvIVS alleles are present in dahlia. Further analysis is required, but in view of the observation that cultivars carrying Type 1 promoter are almost all deeply colored cultivars, it is suggested that this Type 1 promoter–mRNA-1 allele is the allele responsible for deeply colored cultivars. The finding that the anthocyanin contents or flower color intensities in cyanic cultivars are determined by variation in only one gene, DvIVS, is an unexpected result. It is due to high polyploidy in dahlia that the DvIVS Type 1 promoter may have been unintentionally selected for breeding as a color regulation factor.

In conclusion, we suggest that the genotype of DvIVS acts as a key factor determining flower color intensity in dahlia by controlling anthocyanin content via the regulation of anthocyanin pathway genes. In many floricultural species, a change in flower color is caused by mutation in specific structural genes. However, due to the presence of genetic redundancy in highly polyploid plants such as dahlia, a mutation in one specific structural gene rarely affects its phenotype. With the ongoing elucidation of flower color regulation in various species, these results may be used for efficient breeding of highly polyploid crops by marker-assisted selection or genetic modification.
Supplementary Data

**Fig. S1** The observation of petal sections.

**Fig. S2** Relative expression levels of *DvMYB1*, *DvMYB2*, *DvR3MYB*, *DvDEL*, and *DvWDR1*.

**Fig. S3** Alignment of deduced amino acid sequences encoded by *DvIVS* mRNA-1 to mRNA-4.

**Table S1.** Primers used for real-time RT-PCR.

**Table S2.** Primers used for inverse PCR and transcript sequencing.

**Table S3.** Primers used for analyzing the *DvIVS* promoter type.

**Table S4.** Primers used for analyzing the *DvIVS* mRNA type.

**Table S5.** Primers used for analyzing the combination of promoter and mRNA type.

**Table S6.** Measurement of color hue and cellular pH.

Figure legends

**Fig. 1** Simplified flavonoid synthesis pathways in dahlia according to Ohno *et al.* (2011a). The genes framed with a rectangle are regulated by bHLH transcription factor *DvIVS*. Accumulation of anthocyanins and flavones resulted in purple or pink; that of anthocyanins, flavones, and buteins in red; and that of flavones alone in ivory white flower color. Higher anthocyanin contents confer deeper color intensities. In black cultivars, only anthocyanins are accumulated (Deguchi *et al.*, 2013). Abbreviations: ANS, anthocyanidin synthase; CH3H, chalcone 3-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3’H, flavonoid 3’-hydroxylase; FNS, flavone synthase


**Fig. 3** Distribution of L* (Lightness) and c* (Chroma) values of petals in twelve cultivars carrying flavones or anthocyanins and flavones. Squares, triangles, circles, and stars indicate ivory white, pink, purple, and deep purple cultivars, respectively
Fig. 4
Anthocyanin content analysis by spectrophotometer. a: Anthocyanin contents of cultivars. b: Correlation between L* (Lightness) and the reciprocal of anthocyanin content. Squares, triangles, circles, and stars indicate ivory white, pink, purple, and deep purple cultivars, respectively

Fig. 5
Relative expression levels of DvCHS1, DvCHS2, DvCHI, DvF3H, DvDFR, DvANS, and DvIVS. The constitutively expressed gene for actin was used as internal standard. All plots are based on a value of unity for ‘Yukino.’ The data are shown as an average of two biological replications

Fig. 6
Correlation analysis between anthocyanin contents and expression of genes involved in anthocyanin synthesis (a: DvCHS1, b: DvCHS2, c: DvCHI d: DvF3H, e: DvDFR, f: DvANS and g: DvIVS). Each relative gene expression is shown in Fig. 5. Squares, triangles, circles, and stars indicate ivory white, pink, purple, and deep purple cultivars, respectively

Fig. 7
Correlation analysis between DvIVS and anthocyanin synthesis structural gene (a: DvCHS1, b: DvCHS2, c: DvCHI, d: DvF3H, e: DvDFR and f: DvANS) expression in Fig. 5. Squares, triangles, circles, and stars indicate ivory white, pink, purple, and deep purple cultivars, respectively

Fig. 8
Polymorphisms in the DvIVS promoter region. a: PCR polymorphisms in promoter region by IVS-113R and IVS-pro-2F, IVS-pro-3F, or IVS-pro-4F primers (Supplementary Table S3). b: Summary figure based on sequence of DvIVS promoter region. Type 1 is according to the sequence ‘Super Girl’, ‘Yukino’, ‘Cupid’, ‘Evelyn Rumbold’ and ‘Atom.’ Type 2 is according to the sequence ‘Jyunn-ai’, ‘Saffron’ and ‘Hakuyo’, and Type 3 is according to ‘Magokoro’. c: The single-nucleotide polymorphism in the putative TATA-box region of DvIVS. All sequenced cultivars retained a polymorphism.

Fig. S1
The observation of petal sections. Representative fresh petal sections (a-d) and resin sections (e-h) of some cultivars are shown. a, e: ‘Super Girl’, b, f: ‘Evelyn Rumbold’, c, g: ‘Jyunn-ai’, d, h: ‘Gitt’s Attention’. Bars in e-h panels indicate 100 µm. i: Correlation analysis between L* and vertical–horizontal ratio of cell shape. Squares, triangles, circles, and stars indicate ivory white, pink, purple, and deep purple cultivars, respectively
Fig. S2
Relative expression levels of *DvMYB1*, *DvMYB2*, *DvR3MYB*, *DvDEL*, and *DvWDR1*. The constitutively expressed gene for actin was used as internal standard. All plots are based on a value of unity for ‘Yukino.’ The data are shown as an average of two biological replications.

Fig. S3
Alignment of deduced amino acid sequences encoded by *DvIVS* mRNA-1 to mRNA-4. The basic helix-loop-helix domain is shown below the black boxes.

References


Bai Y, Pattanaik S, Patra B, Werkman JR, Xie CH, Yuan L (2011) Flavonoid-related basic helix-loop-helix regulators, *NtAn1a* and *NtAn1b*, of tobacco have originated from two ancestors and are functionally active. Planta 234:363-375


Bate-Smith EC, Swain T (1953) The isolation of 2′,4,4′-trihydroxychalcone from yellow varieties of *Dahlia variabilis*. Journal of the Chemical Society: 2185-2187


Lawrence WJC (1929) The genetics and cytology of Dahlia species. Journal of Genetics 21:125-159


### Table 1. Flavonoid compositions of petals used in this experiment

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Table 2: Putative promoter-mRNA combination inferred from PCR analyses.
Figures

Fig. 1

DvFNS → \text{apigenin (Flavone)}

DvFNS → \text{naringenin (Flavanone)}

DvF3'H \quad \text{DvF3'H}

DvF3'H \quad \text{DvF3'H}

\text{DvFR} \quad \text{leucopelargonidin (Leucoanthocyanidin)}

\text{DvFR} \quad \text{leucocyanidin (Leucoanthocyanidin)}

\text{DvANS} \quad \text{pelargonidin (Anthocyanidin)}

\text{DvANS} \quad \text{cyanidin (Anthocyanidin)}

\text{DvCHS1} + \text{CHR}

\text{DvCHS2} \quad \text{tetrahydroxychalcone (Chalcone)}

4-coumaroyl-CoA + 3 x malonyl-CoA

\text{DvCHS1} \quad \text{DvCHS2}

\text{DvCHI}

\text{DvF3'H}

\text{CH3H} \quad \text{butein}

\text{CH3H} \quad \text{buterin}

\text{eriodictyol (Flavanone)}

\text{dihydroquercetin (Dihydroflavonol)}

\text{dihydrokaempferol (Dihydroflavonol)}

\text{luteolin (Flavone)}
Fig. 3

1. ‘Super Girl’
2. ‘Yukino’
3. ‘Cupid’
4. ‘Evelyn Rumbold’
5. ‘Atom’
6. ‘Magokoro’
7. ‘Jyunn-ai’
8. ‘Saffron’
9. ‘Gitt’s Attention’
10. ‘Zannsetsu’
11. ‘Hakuba’
12. ‘Hakuyo’
Fig. 4

(a) Anthocyanin content per 100 mg fresh petals (mg)

(b) $L^*$ (Lightness)

Cultivars

$r = 0.96^{**}$

1/Anthocyanin content per 100 mg petals
Fig. 5
Fig. 6

(a) Anthocyanin content (mg/100 mg petal) vs. Relative DvCHS1 expression

(b) Anthocyanin content (mg/100 mg petal) vs. Relative DvCHS2 expression

(c) Anthocyanin content (mg/100 mg petal) vs. Relative DvCHI expression

(d) Anthocyanin content (mg/100 mg petal) vs. Relative DvF3H expression

(e) Anthocyanin content (mg/100 mg petal) vs. Relative DvDFR expression

(f) Anthocyanin content (mg/100 mg petal) vs. Relative DvANS expression

(g) Anthocyanin content (mg/100 mg petal) vs. Relative DvIVS expression

Correlation coefficients:
- Fig. 6a: r = 0.97**
- Fig. 6b: r = 0.09
- Fig. 6c: r = 0.15
- Fig. 6d: r = 0.87**
- Fig. 6e: r = 0.90**
- Fig. 6f: r = 0.96**
Fig. 8

a

Deep Purple  Purple  Pink  Ivory White

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Type1  ○ ○ ○ ○ ○ ○ × × × × ×
Type2  × × ○ ○ ○ ○ ○ ○ × × ○
Type3  × × ○ × × ○ ○ ○ ○ × × ×

b

Promoter region

5' UTR

Type1 promoter

Type2 promoter

Type3 promoter

C

TATAbox

Type1 promoter -33 TAACTTAG -26
Type2 promoter -33 TAACTTAG -26
Type3 promoter -33 TAACTTAG -26
## Table S1. Primers used for real-time RT-PCR

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Table S2. Primers used for inverse PCR and transcript sequencing

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<td>IVS-4F</td>
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<td>'Super Girl'</td>
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<td>18.9 ± 0.7</td>
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<td>'Cupid'</td>
<td>23.3 ± 1.4</td>
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<tr>
<td>'Evelyn Rumbold'</td>
<td>24.9 ± 1.1</td>
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<tr>
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<td>58.1 ± 2.2</td>
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<td>'Jyunn-ai'</td>
<td>60.1 ± 1.9</td>
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<td>'Saffron'</td>
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<td>'Gitt's Attention'</td>
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<td>'Red Velvet'</td>
<td>39.9 ± 2.9</td>
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The data shown indicates the mean ± S. E., n = 3 replicates.
Supplemental figures

Fig. S1

1. ‘Super Girl’
2. ‘Yukino’
3. ‘Cupid’
4. ‘Evelyn Rumbold’
5. ‘Atom’
6. ‘Magokoro’
7. ‘Jyunn-ai’
8. ‘Saffron’
Fig. S2

Relative expressions for different cultivars:

- **DvMYB1**
- **DvMYB2**
- **DvR3MYB**
- **DvDEL**
- **DvWDR1**

Cultivars:

- Super Girl
- Yukino
- Cupid
- Evelyn Rumbold
- 'Albatross'
- 'Magikoro'
- 'Journal'
- 'Saffron'
- 'Girl's Amusement'
- 'Zammita'
- 'Relisha'
- 'Volcano'