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A bHLH transcription factor, *DvIVS*, is involved in regulation of anthocyanin synthesis in dahlia (*Dahlia variabilis*)

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Abstract

Dahlias (*Dahlia variabilis*) exhibit a wide range of flower colours because of accumulation of anthocyanin and other flavonoids in their ray florets. Two lateral mutants were used that spontaneously occurred in 'Michael J' (MJW) which has yellow ray florets with orange variegation. MJOr, a bud mutant producing completely orange ray florets, accumulates anthocyanins, flavones, and butein, and MJY, another mutant producing completely yellow ray florets, accumulates flavones and butein. Reverse transcription–PCR analysis showed that expression of *chalcone synthase 1* (*DvCHS1*), *flavanone 3-hydroxylase* (*DvF3H*), *dihydroflavonol 4-reductase* (*DvDFR*), *anthocyanidin synthase* (*DvANS*), and *DvIVS* encoding a basic helix–loop–helix transcription factor were suppressed, whereas that of *chalcone isomerase* (*DvCHI*) and *DvCHS2*, another *CHS* with 69% nucleotide identity with *DvCHS1*, was not suppressed in the yellow ray florets of MJY. A 5.4 kb CACTA superfamily transposable element, *transposable element of Dahlia variabilis 1* (*Tdv1*), was found in the fourth intron of the *DvIVS* gene of MJW and MJY, and footprints of *Tdv1* were detected in the variegated flowers of MJW. It is shown that only one type of *DvIVS* gene was expressed in MJOr, whereas these plants are likely to have three types of the *DvIVS* gene. On the basis of these results, the mechanism regulating the formation of orange and yellow ray florets in dahlia is discussed.

Key words: Anthocyanin, bHLH, CACTA superfamily, dahlia, transcription factor.

Introduction

Dahlias (*Dahlia variabilis*) are popular ornamental plants cultivated in many countries. They belong to the Asteraceae and are autoalloctoploids with chromosome number $2n=64$ (Lawrence, 1929, 1931*b*; Lawrence and Scott-Honcrieff, 1935; Gatt *et al.*, 1998). Because of high polyploidy, dahlias exhibit various inflorescence shapes, colours, and sizes, and ~50 000 cultivars have been identified and named in the past century (McClaren, 2009). In particular, dahlias exhibit

a wide range of ray floret colours, such as ivory, yellow, pink, red, purple, and black. The pigments accumulated in ray florets are flavonoids, mainly anthocyanins, butein, and flavones and their derivatives that produce red, yellow, and ivory colours, respectively (Bate-Smith and Swain, 1953; Nordström and Swain, 1953, 1956, 1958; Bate-Smith *et al.*, 1955; Harborne *et al.*, 1990; Yamaguchi *et al.*, 1999). In previous studies, four elements were believed to explain the

inheritance of ray floret colours in dahlia: A (pale anthocyanin), B (deep anthocyanin), I (flavone), and Y (yellow) (Lawrence, 1931a; Lawrence and Scott-Honcrieff, 1935; Bate-Smith *et al.*, 1955; Broertjes and Ballego, 1967; Singh *et al.*, 1970). Despite its importance, few studies use molecular analysis techniques such as gene isolation and enzyme analysis (Fischer *et al.*, 1988; Wimmer *et al.*, 1998; Yamaguchi *et al.*, 1999; Ogata *et al.*, 2001; Suzuki *et al.*, 2002). Flavonoid synthesis pathway genes have not yet been isolated, except for flavonoid 3′-hydroxylase (Schlangen *et al.*, 2009, 2010a).

The flavonoid synthesis pathway is the most well-studied secondary metabolite synthesis pathway in plants. Since flavonoids determine flower colours, regulation of the flavonoid synthesis pathway is very important in ornamental plants. In particular, anthocyanins are found in the flowers of many species and have many functions, such as recruiting pollinators and seed dispersers and scavenging active oxygen species (Yamasaki *et al.*, 1996; Winkel-Shirley, 2001). Anthocyanidin, the aglycone of anthocyanin, is formed in the anthocyanin synthesis pathway by the condensation of three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA (Fig. 1); the enzymes involved in this pathway are as follows: chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS). The synthesized anthocyanidins are glycosylated by glucosyltransferase and modified through processes such as acylation and malonylation (Tanaka *et al.*, 2008). The genes encoding the above-mentioned enzymes involved in the anthocyanin synthesis pathway are regulated by transcription factors such as basic helix–loop–helix (bHLH), R2R3-MYB,

and WD40 repeats (WDRs); these three proteins function as transcription factors by forming complexes or acting alone (Koes *et al.*, 2005). In *D. variabilis*, first, three molecules of malonyl-CoA and one molecule of 4-coumaroyl-CoA, the same substrate as that in chalcone synthesis, are converted to trihydroxychalcone by CHS and chalcone reductase (Bomati *et al.*, 2005) and then hydroxylated by chalcone 3-hydroxylase or a particular copy of flavonoid 3′-hydroxylase to form butein. Recently, the gene for chalcone 3-hydroxylase, which converts trihydroxychalcone to butein, was isolated from *Cosmos sulphureus* (Schlangen *et al.*, 2010b), but whether the anthocyanin and butein synthesis pathways are regulated by the same regulator gene is unknown.

To isolate the genes that impart colour to flowers, mutants formed by transposable elements have been widely used. This method of gene isolation, called transposon tagging, was used to isolate *Antirrhinum majus pallida* (*DFR*) (Martin *et al.*, 1985), *Zea mays bronze* (*UDP-glucose flavonoid 3-O-glucosyltransferase*) (Fedoroff *et al.*, 1984), *Petunia hybrida ph6* (*An1*) (Chuck *et al.*, 1993), and many more. Therefore, to clarify whether anthocyanin and butein synthesis in dahlia are regulated by the same or different mechanisms, two lateral mutants MJOr and MJY that spontaneously occurred in 'Michael J' (MJW), which has yellow ray florets with orange variegation, were used. MJOr, a bud mutant producing orange ray florets, accumulates anthocyanins, flavones, and butein, and MJY, another mutant producing yellow ray florets, accumulates flavones and butein. In this study, anthocyanin synthesis pathway genes and transcription factors were isolated and it was shown that DvIVS, belonging to the An1 subgroup bHLH transcription factor family, regulates anthocyanin synthesis in ray florets of dahlia. Finally, a putative model to explain the mechanism regulating expression of orange and yellow flower colours in dahlia is proposed.
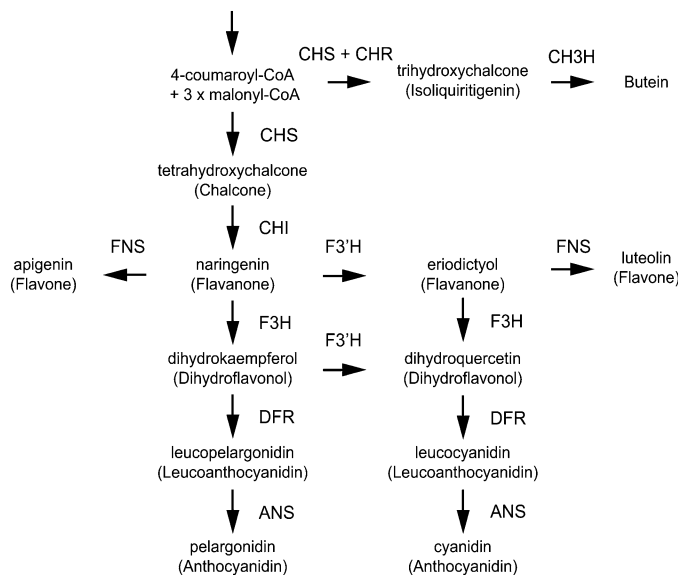
Materials and methods

Plant material and developmental stages

Dahlia variabilis cv. 'Michael J' (MJW) was obtained from Yukihiro Fukuda (Haibara, Nara, Japan). MJW has bright yellow ray florets with a small number of brilliant orange markings (Fig. 2A). It rarely produces completely orange (Fig. 2B) or completely yellow (Fig. 2C) ray florets as a bud mutation. The completely orange mutant line (MJOr) and the completely yellow ray floret mutant line (MJY) were isolated and obtained from Yukihiro Fukuda. The two lines and wild-type plants were grown under standard greenhouse conditions or in the experimental field of Kyoto University (Kyoto, Japan). To analyse temporal gene expression, the MJOr ray florets were classified into five stages on the basis of the degree of colouration (Fig. 3A): stage 1, an uncoloured ray floret; stage 2, the tip of a ray floret is coloured; stage 3, a ray floret coloured in the centre; stage 4, colouring is completed and the ray floret starts to unfold; and stage 5, a completely unfolded ray floret. The size and stage of MJY ray florets used were almost same.

HPLC analysis

Ray florets were soaked overnight in 5 ml of MAW solution (methanol:acetic acid:water, 4:1:5 v/v/v) to extract the pigments.

Fig. 1. Simplified flavonoid synthesis pathways. Only compounds detected in 'Michael J' are shown. 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.
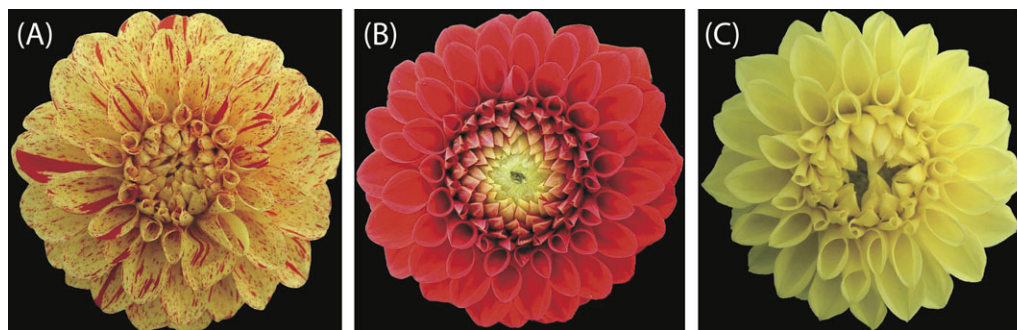
Fig. 2. Inflorescence phenotypes of 'Michael J' (MJW) and its bud mutants: (A) MJW, (B) MJOr, and (C) MJY. (A) MJW ray florets are bright yellow with a small number of brilliant orange markings. (B) MJOr ray florets are brilliant orange; this colour is mainly derived from anthocyanins and butein. (C) MJY ray florets are bright yellow; this colour is mainly derived from butein.
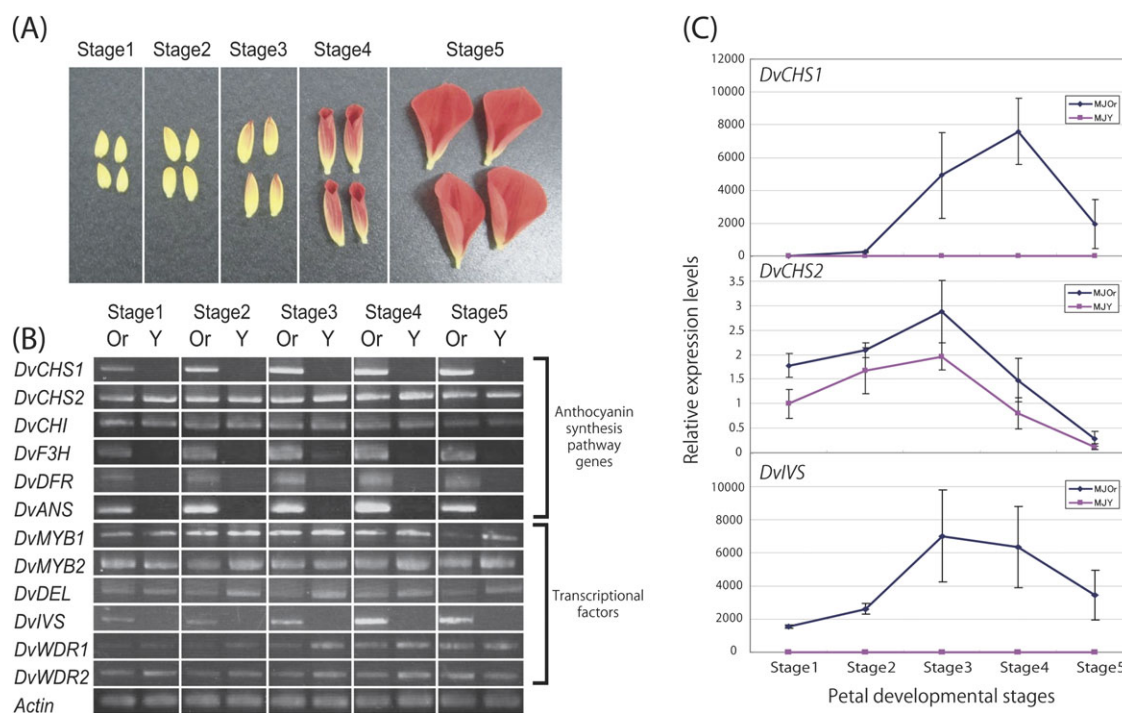

Fig. 3. Temporal expression of anthocyanin synthesis pathway genes and transcription factors. (A) Developmental stages of ray florets. Developmental stages of MJOr ray florets were defined on the basis of the degree of colouration. (B) Semi-quantitative RT-PCR analysis of temporal expression of anthocyanin synthesis genes and transcription factors in MJOr and MJY. The constitutively expressed gene for actin in *Dahlia variabilis* was used as an internal control. (C) Relative expression levels of *DvCHS1, DvCHS2*, and *DvIVS* in MJOr compared with those in MJY at each ray floret developmental stage. The vertical bars are the standard error ($\pm$SE) of the means of three biological replicates. The constitutively expressed gene for actin in *D. variabilis* was used as an internal control.

For pigment hydrolysis, extracted solutions were evaporated, redissolved in 2 ml of 20% hydrochloric acid, boiled, and used as crude aglycones. 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 photo-diode array detector. The detection wavelength was 350 nm for flavones, 380 nm for chalcones, and 530 nm for anthocyanins. Elutant A was 1.5% phosphate dissolved in water and elutant B was 1.5% phosphate, 20% acetic acid, and 25% MeCN dissolved in water. Analysis was performed at a flow rate of 1 ml min^{-1} and column temperature of 40 °C, using a mobile phase gradient starting at 20% B to 85% B over 40 min with 5 min re-equilibration at 20% B. As standards for the determination of flavonoids, commercially available naringenin, apigenin, and luteolin (Wako Pure Chemical Industries Ltd, Osaka, Japan) as well as HPLC-separated and HPLC-purified hydrolysed cyanidin

and pelargonidin from rose petals were used. To obtain hydrolysed standards of butein and isoliquiritigenin, extracts from orange ray florets of a seedling line, HywR7R, were separated by paper chromatography, and each band was eluted with methanol. Each eluate was dried and redissolved in a small amount of methanol. The colour, R_F value, and maximum wavelength of eluted compounds were measured, and the compounds were determined by comparing the data with those of authentic butein (kindly supplied by Dr Norio Saito) and previously reported data (Nordström and Swain, 1956; Saito *et al.*, 1970).

Isolation of nucleic acids and sequencing

To clone the *DvIVS* genomic region and *Tdv1* region, genomic DNA of ray florets was isolated using a modified cetyltrimethyl-ammonium bromide method (Murray and Thompson, 1980) and

purified with MagExtractor™-Plant Genome (Toyobo Co. Ltd, Osaka, Japan). Genomic DNA was also isolated using Qiagen Genomic-tip 100/G (Qiagen, Valencia, CA, USA) for genomic PCR analyses. Total RNA was isolated using the isothiocyanate/ caesium chloride centrifugation method and an RNeasy Plant Mini Kit (Qiagen), a modified QuickGene RNA Cultured Cell Kit S (Fujifilm, Tokyo, Japan), or a Get pure RNA Kit (Dojindo, Kumamoto, Japan). All sequence analyses were performed using a BigDye® Terminator v 3.1 Cycle Sequencing Kit and a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Gene isolation

DvCHS1, *DvCHI*, *DvF3H*, *DvDFR*, *DvANS*, *DvIVS*, *DvDEL*, and *DvActin* cDNAs were isolated using degenerate primers designed from conserved regions of each gene shown in Supplementary Table S1 available at *JXB* online. To isolate *DvMYB1*, *DvMYB2*, *DvWDR1*, and *DvWDR2* cDNAs, dahlia cDNA libraries provided by Dr Yoshikazu Tanaka (Suntory, Shimamoto, Mishima, Osaka, Japan) were screened (Suzuki *et al.*, 2002). *Ipomoea nil* MYB (AB232770) and WDR (AB232779) cDNAs were used as probes, and AlkPhos Direct (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was used for probe hybridization. To determine the complete sequence of mRNA of all genes, 3' rapid amplification of cDNA ends (RACE) and 5' RACE was performed using a GeneRacer™ Kit (Invitrogen, Carlsbad, CA, USA). Primers used are shown in Supplementary Table S2. Each end of the genes was sequenced for at least 16 clones for each gene to avoid single nucleotide polymorphisms (SNPs) because of dahlia's high polyploidy. While performing 5' RACE of *DvMYB1*, a novel CHS-like fragment was obtained that differed from *DvCHS1*. This new CHS was named *DvCHS2* and each cDNA end was determined by 5' and 3' RACE. For 5' and 3' RACE of the transcription factor genes, total RNA extracted from *D. variabilis* 'Matsuribayashi' was used.

RT-PCR and real-time RT-PCR

The total RNA of MJOr and MJY was subjected for reverse transcription using an oligo(dT)$_{20}$ primer and ReverTra Ace (Toyobo). The obtained cDNA products served as templates for PCR performed using Blend *Taq* polymerase (Toyobo). *DvActin* was used as an internal standard. The primers used are shown in Supplementary Table S3 at *JXB* online. The PCR program was set at 94 °C for 2 min, followed by 30–35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2–3 min.

Total RNA extracted from stage 1–5 ray florets was used. Real-time RT-PCR was performed using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Ohtsu, Japan) according to the manufacturer's instructions. Real-time PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The primers used are shown in Supplementary Table S4 at *JXB* online. The PCR program was set at 50 °C for 1 s, 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s, 72 °C for 20 s, and the subsequent dissociation steps. Three different flowers were used as replicates for each stage, and *DvActin* was used as an internal standard. Because of the low transcription level, samples that did not reach the threshold were calculated as 40 cycles.

To test partial expression of *DvIVS*, three primer sets were used for RT-PCR: IVS-Full-F and IVS-Full-R, 35S-IVS-F and IVS-611R, and IVS-725F and IVS-Full-R (Supplementary Table S5 at *JXB* online). The first primer set amplifies from the start to the stop codon. The second primer set amplifies from 30 bp after the start codon to the third exon, and the third primer set amplifies from the fifth exon to the stop codon.

Cloning of the genomic DvIVS gene and the transposable element

Genomic PCR was performed with LA Taq (Takara) using 100 ng of genomic DNA 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. Amplified PCR products were electrophoresed with 1× TAE and a 0.8% agarose gel, and cloned into pCR®-XL-TOPO using a TOPO® XL PCR Cloning Kit (Invitrogen) according to the manufacturer's instructions. The *DvIVS* gene of MJOr was amplified using the primer set IVS-Full-F and IVS-Full-R (Supplementary Table S5 at *JXB* online). The insertion sequence of MJY was amplified with the primers IVS-G2241F and IVS-G2869R (Supplementary Table S5), which were designed on the basis of the upstream and downstream regions of the insertion sequence. Four plasmids with the *DvIVS* gene and six plasmids with inserted fragments were sequenced by primer walking using the primers shown in Supplementary Table S5.

Sequence analysis of cDNAs and the genomic DvCHS1 gene

To examine regulation of redundant copies of each anthocyanin synthesis gene, sequence analyses for multiple copies of these genes were performed. Extracted total RNAs of MJOr and MJY were subjected to reverse transcription with an oligo(dT)$_{20}$ primer using ReverTra Ace (Toyobo). RT-PCRs of *DvCHS1*, *DvCHI*, *DvF3H*, and *DvDFR* were performed using KOD Plus polymerase (Toyobo), and PCR products were cloned into pCR®-Blunt II-TOPO® using a Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). The PCR program was set at 94 °C for 2 min, followed by 30 cycles of 94 °C for 50 s, 55 °C for 30 s, and 68 °C for 2 min. A total of 40 cloned plasmids of both lines with *DvCHS1*, *DvCHI*, *DvF3H*, and *DvDFR* were extracted, except for MJY *DvCHS1* (only 20 plasmids were extracted). To determine the accurate sequence, plasmids with *DvCHS1*, *DvF3H*, and *DvDFR* of both lines were sequenced twice from both the 3' and 5' ends. The primers used are shown in Supplementary Table S6 at *JXB* online. To exclude misreading of sequences or misamplification in PCR, SNPs that were only observed in one clone were omitted.

To analyse the sequence of the genomic region of *DvCHS1* in MJOr, MJY, and MJW, genomic PCR was performed using the primer set shown in Supplementary Table S3 at *JXB* online. A total of 30 clones of the *DvCHS1* gene were sequenced for each line.

Denaturing gradient gel electrophoresis (DGGE) analysis

RT-PCR was performed with KOD Dash polymerase (Toyobo), using the cDNA of MJOr and MJY. The PCR program was set at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 10 s, and 74 °C for 30 s. The primers used were DGGE-F (5'-CCCGGGCGGGCCCGCGATCTTGGA-3') and DGGE-R (5'-GAATGTTTCAAGCACACATTGCTT-3'). After confirming a single band by agarose gel electrophoresis, perpendicular DGGE was performed using a DGGE Mini Electrophoresis System (NIHON EIDO Co. Ltd, Tokyo, Japan). To make the gradient gel, 35 ml of 0% denatured 6.5% acrylamide solution with 190 µl of 10% APS and 16 µl of TEMED was added to 35 ml of 80% denatured 6.5% acrylamide solution with 190 µl of 10% APS and 16 µl of TEMED in the gradient chamber. The mixed gel solution was gradually poured into the gradient gel electrophoresis tank (1.8–2 ml min^{-1}) using a solution dispensing pump. An aliquot of 50 µl of the PCR product was applied to the gel and electrophoresed with 1× TBE buffer for 4 h at 80 V and 60 °C. The gel was stained with SYBR Gold (Invitrogen), and the band was confirmed by UV exposure.

GenBank accession numbers

Sequence data of these genes have been deposited in the DDBJ/ GenBank data libraries under accession numbers AB576129, AB576130, AB576131, AB576132 (*DvCHS1*), AB576133 (*DvCHS2*), AB591827 (*DvCHI*), AB591828 (*DvF3H*), AB591829 (*DvDFR*), AB591830 (*DvANS*), AB601003 (*DvMYB1*), AB601004 (*DvMYB2*), AB601005 (*DvIVS*), AB601006 (*DvDEL*), AB601007

(*DvWDR1*), AB601008 (*DvWDR2*), AB601010 (*DvIVS* genomic region), and AB601009 (*Tdv1*).

Results

Pigment analysis

Wild-type MJW produces yellow ray florets with orange variegation, while MJOr and MJY produce completely orange and completely yellow ray florets, respectively, without any variegation. To clarify the difference in pigments between MJOr and MJY, aglycones were extracted from ray florets and analysed by HPLC. In both lines, peaks of chalcones (butein and isoliquiritigenin) and flavones (apigenin and luteolin) were detected. In addition to these, peaks of anthocyanidins (pelargonidin and cyanidin) were detected in MJOr but not in MJY (Table 1). These data showed that chalcones and flavones are synthesized in both lines, while anthocyanidins are synthesized only in MJOr.

Temporal expression of anthocyanin synthesis pathway genes

To analyse genes associated with anthocyanin synthesis, partial sequences of *DvCHS1*, *DvCHI*, *DvF3H*, *DvDFR*, *DvANS*, *DvIVS*, and *DvDEL* were isolated using degenerate primers. *DvMYB1*, *DvMYB2*, *DvWDR1*, and *DvWDR2* were isolated by cDNA library screening using cDNA fragments of *InMYB1* and *InWDR*1 as probes (Morita *et al.*, 2006). Full cDNA sequences of all genes including *DvCHS2*, another *CHS* gene, were isolated by 5′ and 3′ RACE. Partial sequences of *DvActin* were also isolated with degenerate primers by 3′ RACE.

To examine the temporal expression pattern of the anthocyanin synthesis pathway genes during ray floret development, the MJOr ray florets were divided into five developmental stages based on the degree of colouration and RT-PCR was performed. Although expression levels of *DvCHS2* and *DvCHI* showed no difference, expression levels of *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS* were lower in MJY than in MJOr at all developmental stages (Fig. 3B). Down-regulation of multiple genes encoding the anthocyanin synthesis pathway enzymes suggested that transcription factor(s) do not function in MJY.

Temporal expression analysis by RT-PCR was also performed for transcription factors. Although expression levels of *DvMYB1*, *DvMYB2*, *DvDEL*, *DvWDR1*, and *DvWDR2* did not differ significantly, the expression level of *DvIVS* was lower in MJY than in MJOr, which is similar to that of *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS* (Fig. 3B). This result suggested that *DvIVS* is a transcription factor that activates anthocyanin biosynthesis genes including *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS*.

To confirm these results, real-time RT-PCR of *DvCHS1* and *DvCHS2*, which seem to be regulated and not regulated by *DvIVS*, respectively, was performed using total RNA extracted from stage 1–5 ray florets. In MJOr, the relative expression level of *DvIVS* and *DvCHS1* compared with that of *DvActin* increased until stage 3 or 4 and then decreased, but consistently very low expression levels were maintained in MJY (Fig. 3C). In contrast, expression levels of *DvCHS2* were slightly lower in MJY, but the expression pattern of *DvCHS2* in MJOr and MJY was the same (Fig. 3C). This result supported the results of RT-PCR analyses and suggested that *DvCHS1*, but not *DvCHS2*, is regulated by DvIVS.

DvCHS1 (Supplementary Table S7 at *JXB* online), *DvCHI* (Supplemenary Table S8), *DvF3H* (Supplementary Table S9), and *DvDFR* (Supplementary Table S10) cDNA sequences isolated from MJOr and MJY ray florets were determined and the number of different sequences expressed in both lines was determined on the basis of SNPs. Based on sequence analysis of transcripts, 17 and 15 sequences of *DvCHI* (Supplementary Table S8), nine and 12 sequences of *DvF3H* (Supplementary Table S9), and nine and eight sequences of *DvDFR* (Supplementary Table S10) were observed in MJOr and MJY, respectively. Because drastic differences were not observed in the number of sequences expressed in both lines, whole copies were assumed to be suppressed in MJY. However, 12 different sequences of *DvCHS1* were detected in MJOr, while only one sequence was detected in MJY (Supplementary Table S7). At least three major transcripts (*DvCHS1* type 1, 2, and 4) were detected in MJOr, while only one transcript (*DvCHS1* type 4) was detected in MJY (Supplementary Table S7). The genomic fragments of *DvCHS1* were isolated and it was observed that not only MJOr and MJW, but also MJY has *DvCHS1* genes that have coding sequences identical to these three major transcripts (Supplementary Tables S11, S12).

Characterization of DvIVS

The full-length *DvIVS* cDNA was 2483 bp containing a 649 amino acid open reading frame (ORF). The *DvIVS* ORF retained the bHLH domain at 472–530 amino acids (Fig. 4A). BLASTP search (http://blast.ncbi.nlm.nih.gov/

Table 1. Aglycones in the petals of MJOr and MJY

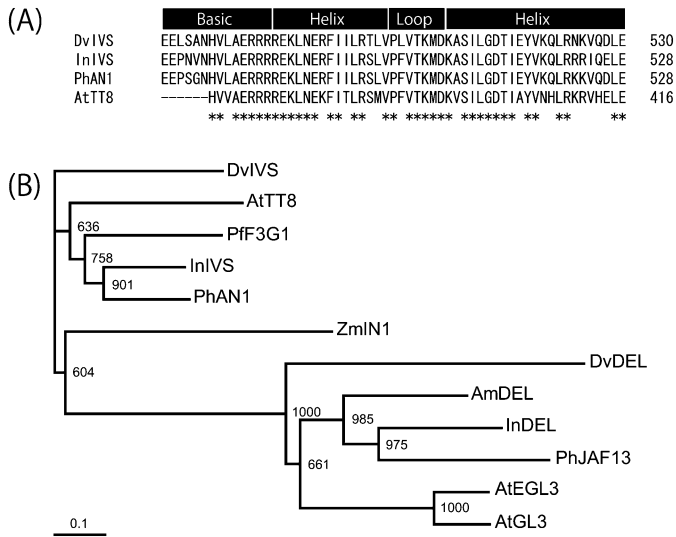| Line	Anthocyanidin		Flavone		Chalcone	
	Pelargonidin	Cyanidin	Apigenin	Luteolin	Butein	Isoliquiritigenin
Orange	+	+	+	+	+	+
Yellow	−	−	+	+	+	+

+, Abundant; −, not detected.

(A)

(B)

Fig. 4. Phylogenetic analysis of DvIVS. (A) Amino acid comparison of the bHLH domain of *DvIVS, InIVS, PhAN1*, and *AtTT8*. Numbers (*) indicate amino acids that are fully conserved in each of the proteins. (B) Phylogenetic tree for bHLH transcription factors associated with anthocyanin synthesis pathways. The entire amino acid sequences were aligned using ClustalW, and the tree was constructed by the Neighbor–Joining method. Bootstrap values of 1000 retrials are indicated on each branch, and the scale shows 0.1 amino acid substitutions per site. The abbreviations shown in front of each protein indicate the plant species: Dv, *Dahlia variabilis*; Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; In, *Ipomoea nil*; Pf, *Perilla frutescens*; Ph, *Petunia hybrida*; Zm, *Zea mays*. The accession number of each protein is: DvIVS (BAJ33515), DvDEL (BAJ33516), AmDEL (AAA32663), AtEGL3 (NP_176552), AtGL3 (NP_680372), AtTT8 (CAC14865), InDEL (BAE94393), InIVS (BAE94394), PfF3G1 (BAC56998), PhAN1 (AAG25927), PhJAF13 (AAC39455), and ZmlN1 (AAB03841).

Blast.cgi) revealed that the deduced amino acid sequence was 48% identical to InIVS (*I. nil*), 50% identical to An1 (*Petunia hybrida*), and 48% identical to AtTT8 (*Arabidopsis thaliana*). To compare genetic distance among the bHLH proteins associated with anthocyanin synthesis in higher plants, a phylogenetic tree was constructed. The phylogenetic tree showed that *DvIVS* belonged to the *An1* subgroup (Fig. 4B).

Analysis of the *DvIVS* genomic region

The *DvIVS* genomic region coding for full-length mRNA (AB601010) of MJOr was 8477 bp and contained nine exons and eight introns (Fig. 5A). Genomic PCR was performed to examine whether a transposable element was inserted in the *DvIVS* gene of MJY. An ~8.2 kb amplified product was obtained from MJOr, and an ~13.6 kb amplified product was obtained from MJY (data not shown). The 5385 bp insertion sequence (AB601009) was found in the fourth intron, 903 bp downstream of the fourth exon of the *DvIVS* gene of MJY. To characterize the structure of the *DvIVS* gene of MJW, genomic PCR was performed using the primer set IVS Full-F and IVS-G2869R. Not only the genomic

fragment (8.2 kb) that should include the insertion sequence, but also a shorter fragment (2.8 kb) without the insertion sequence were detected in MJW (Fig. 5A).

The inserted element of MJY carried a terminal inverted repeat (TIR) starting with 5′-CACTA-3′ (Table 2), which is characteristic of a CACTA superfamily transposable element. The insertion generated a 3 bp (TGG) target site duplication (TSD), similar to the TSD generated by other CACTA-type transposable elements (Inagaki *et al.*, 1994; Zabala and Vodkin, 2008; Xu *et al.*, 2010). It also contained an ORF for antisense orientation (Fig. 5A) (nucleotides 1735–3555), encoded a 606 amino acid polypeptide, and shared high identity with other CACTA superfamily transposable elements [41% identical to TNP2 in *A. majus Tam1* (Nacken *et al.*, 1991), 33% identical to TNP2 in *Glycine max Tgm9* (Xu *et al.*, 2010), and 32% identical to *En-1* in *Zea mays* (Pereira *et al.*, 1986)]. To test whether the insertion sequence has the ability to excise from the *DvIVS* gene, 600 bp fragments containing the insertion site in MJW were amplified and cloned into plasmid vectors. A total of 23 clones were obtained with the *DvIVS* gene fragments with small rearrangements that were presumed to be footprint sequences of the insertion sequence (Table 3, Fig. 5B). From these results, the insertion sequence was regarded as an active transposable element and named *transposable element of D. variabilis 1* (*Tdv1*). The *DvIVS* fragments could be obtained from MJOr but not from MJY. In addition to the *DvIVS* gene fragment, two different gene fragments were obtained. Both of them were shorter than the *DvIVS* fragments, and showed 97% identity to the *DvIVS* gene. This suggested that the dahlia lines used here have at least three types of *DvIVS* genes.

To test whether all three *DvIVS* genes are expressed in the flowers, *DvIVS* cDNA fragments from MJOr were amplified, cloned into a plasmid vector, and the cDNA sequence was analysed. Although 20 clones of *DvIVS* cDNA were sequenced, all of them were derived from the *DvIVS* gene (data not shown). The result suggested that the anthocyanin pigmentation in the flowers depend on the *DvIVS* gene and that the two other *DvIVS* genes are not expressed in the flowers.

To characterize the *DvIVS* transcripts in MJY, RT-PCR was performed using three primer sets. Successful amplification was achieved only by the primer set amplifying the region upstream of *Tdv1* insertion sites in both MJOr and MJY; however, other primer sets did not successfully amplify regions in MJY (Fig. 5C). This suggested that *Tdv1* insertion into the fourth intron of *DvIVS* resulted in truncated *DvIVS* mRNA in MJY. On analysing *DvIVS* mRNA expression in MJY by 3′ RACE, truncated *DvIVS* mRNA containing the fourth exon and a short part of the fourth intron (nucleotides 125–250 downstream of the fourth exon/fourth intron junction) was detected (data not shown). Thus, *Tdv1* insertion into the *DvIVS* gene of MJY resulted in generation of short-sized *DvIVS* transcripts lacking the bHLH domain, thereby leading to an inability for transcriptional regulation of anthocyanin synthesis genes.
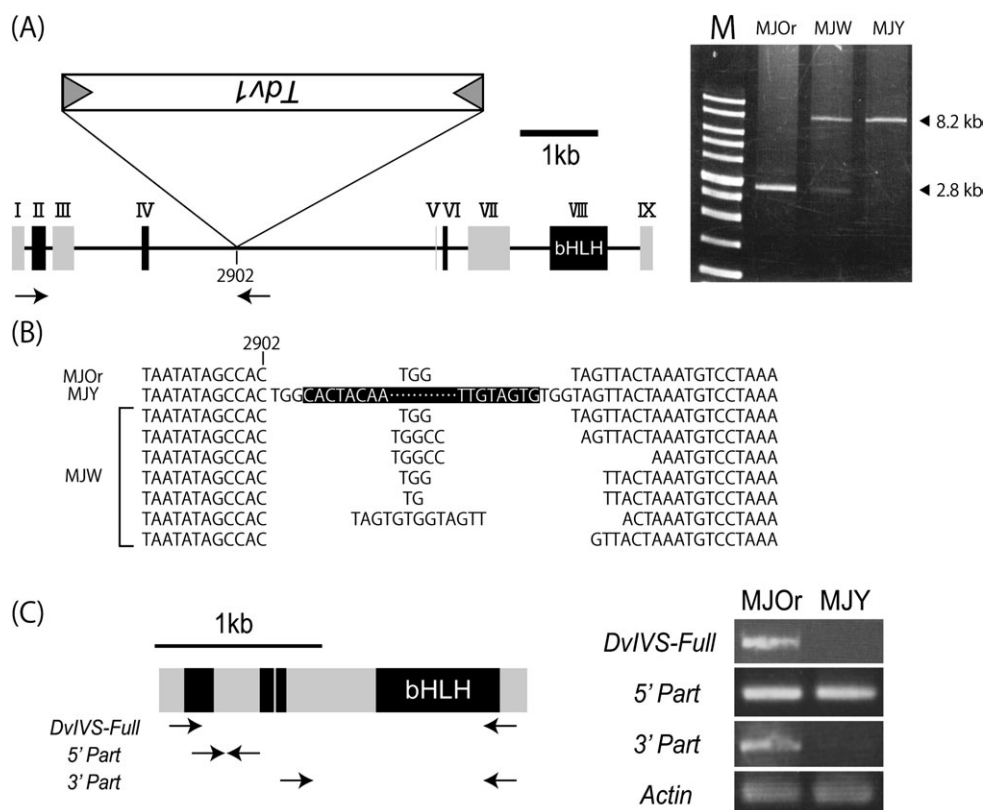
Fig. 5. Structure of the *DvIVS* gene. (A) Structure of the *DvIVS* gene and genomic PCR. Rectangles with roman figures indicate exons. The bHLH domain is located in the eighth exon. *Tdv1* is inserted in the fourth intron in the antisense direction of the MJY genome. Arrows indicate the primer set used for genomic PCR. M indicates a 1 kb marker. (B) Footprints of *Tdv1*. The uppermost sequence is assumed to be the standard sequence. Sequences in the black box indicate the *Tdv1* sequence. With respect to MJW, *Tdv1*-inserted sequences were not analysed. (C) Truncated transcripts of *DvIVS* in MJOr and MJY. Grey rectangles indicate odd numbered exons and black rectangles indicate even numbered exons. Arrows indicate primer sets used for RT-PCR.

Table 2. Comparison of TIRs with the reported transposable elements of the CACTA superfamily

Name	TIR sequences	Length (bp)	Species	Reference
Tdv1	CACTACAA	8	*Dahlia variabilis*	This study
En1	CACTACAAGAAAA	13	*Zea mays*	Pereira *et al.* (1986)
Tam1	CACTACAACAAAA	13	*Antirrhinum majus*	Nacken *et al.* (1991)
Cac1	CACTACAA	8	*Arabidopsis thaliana*	Miura *et al.* (2001)
Tpn1	CACTACAAGAAAAATGCACATAGCAAC	27	*Pharbitis nil (Ipomoea nil)*	Inagaki *et al.* (1994)
Tgm1	CACTATTAGAAAA	13	*Glycine max*	Vodkin *et al.* (1983)
Cs1	CACTATGTGAAAAAAGCTTA	20	*Sorghum bicolor*	Chopra *et al.* (1999)
Pis1	CACTACGCCAAA	12	*Pisum sativum*	Shirsat (1988)
Psl	CACTACAAAAA	12	*Petunia hybrida*	Snowden and Napoli (1998)
Tdc1	CACTACAAGAAACGCGAGA	20	*Daucus carota*	Ozeki *et al.* (1997)
Tnr3	CACTAGAAGGGAT	13	*Oryza sativa*	Motohashi *et al.* (1996)

This table is based on Tian (2006).

Discussion

DvIVS is a bHLH transcription factor associated with anthocyanin synthesis in dahlia

The following transcription regulators of anthocyanin synthesis pathway enzymes are well known: bHLH, R2R3-MYB, and WDR (Koes *et al.*, 2005). To date, many studies have been conducted to determine flavonoid synthesis pathway transcription factors. *An1* and *JAF13*, *An2*, and *An11* have been isolated as bHLH, R2R3-MYB, and WDR transcription factors, respectively, regulating anthocyanin synthesis in petunia (Quattrocchio *et al.*, 1993, 1998; De Vetten *et al.*, 1997; Spelt *et al.*, 2000, 2002). Similarly, bHLH, R2R3-MYB, and WDR transcription factors control anthocyanin synthesis in the three *Ipomoea* species (Park *et al.*, 2004, 2007; Morita *et al.*, 2006).

bHLH proteins are found in both animals and plants, and constitute a superfamily of transcription factors.

Table 3. Amplified fragment with IVS-G2241F and IVS-G2869R

Type	Length (bp)	Identity to the DvIVS genome (%)	MJOr	MJY	MJW
1	628	100	10	0	23[a]
2	625	97	6	16	24
3	615	97	1	3	16

[a] Footprint sequences were not considered in type 1 of MJW.

Arabidopsis has at least 147 bHLH genes, and these are divided into 21 subfamilies (Toledo-Ortiz *et al.*, 2003). The bHLH genes associated with regulation of anthocyanin synthesis were first found in *Z. mays* (Ludwig *et al.*, 1989), and have subsequently been found in many other species. Recent studies showed that *AtTT8* (Nesi *et al.*, 2000), *AtGL3* (Payne *et al.*, 2000), and *AtEGL3* (Zhang *et al.*, 2003) regulate anthocyanin and proanthocyanidin synthesis in *A. thaliana*. These bHLH genes regulating anthocyanin synthesis are usually divided into two subgroups on the basis of their phylogenetic tree (Spelt *et al.*, 2000): one subgroup includes *AtTT8*, *An1*, and *InIVS*, and the other includes *AtGL3*, *AtEGL3*, *JAF13*, and *InDEL*. Usually, the bHLH domain in the former subgroup is not separated by an intron, while that in the latter group is separated by an intron. For example, the bHLH domains of *An1* (Spelt *et al.*, 2000), *IpIVS* (Park *et al.*, 2007), *AtTT8* (Nesi *et al.*, 2000), and *GtbHLH1* in *Gentiana triflora* (Nakatsuka *et al.*, 2008) are encoded within a single exon, while those of *JAF13* and *ZmIn1* are encoded within two exons. *DvIVS* shows high identity with *An1* (50%), *InIVS* (48%), and *AtTT8* (48%) in deduced amino acid sequences and is classified into the same subgroup with these bHLHs in the phylogenetic tree (Fig. 4B). *DvIVS* has a bHLH domain encoded within a single exon (Fig. 5A), suggesting that the structure of the *DvIVS* gene is similar to that of the *An1* gene. These data suggest that *DvIVS* is an orthologue of *AtTT8*, *An1*, and *InIVS*, and that *DvIVS* regulates transcription of *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS*, but not of *DvCHS2* and *DvCHI*. Real-time RT-PCR showed that the expression patterns of *DvIVS* and *DvCHS1* were highly coordinated, while that of *DvCHS2* was not, supporting the suggestion (Fig. 3C). A slight decrease in the expression levels of *DvCHS2* was observed in MJY; this may have been caused by the excess production of substrate resulting from an inability to synthesize anthocyanin. In many cases, although the particular set of genes may differ between species and tissues within a species, a subset of the anthocyanin pathway genes is affected by these transcription factors (Gonzalez *et al.*, 2008). Transcription regulation in the three *Ipomoea* species (Park *et al.*, 2004, 2007; Morita *et al.*, 2006) is similar to that in dahlia.

The cDNA fragment derived from the 5′ end of *DvIVS* mRNA containing the first to third exons was detected in MJY; however, the cDNA fragment from the 3′ end of *DvIVS* mRNA was not observed. This truncated mRNA did not contain a bHLH domain in the eighth exon, suggesting that the bHLH domain of DvIVS is important for anthocyanin synthesis in dahlia.

Flower colour variegation is caused by Tdv1

Flower colour variegation is often caused by recurrent excision of transposable elements in a pigment biosynthesis gene. For example, *Tpn1*, a CACTA superfamily transposable element in *DFR* in *I. nil* (Inagaki *et al.*, 1994); *Tip100*, a *h*AT superfamily transposable element in *CHS-D* in *I. purpurea* (Habu *et al.*, 1998); and *Tgm9*, a CACTA superfamily transposable element in *DFR2* in *G. max* (Xu *et al.*, 2010) resulted in variegated flower phenotypes. Therefore, an association of transposable elements with floret colour was expected in MJW. It was hypothesized that MJOr was a complete revertant mutant line and MJY was a loss-of-function mutant line, where the transposable element could not transpose from a gene associated with anthocyanin synthesis.

A 5.4 kb insertion sequence, named *Tdv1*, was found in the fourth intron of the *DvIVS* gene in MJY. *Tdv1* shares a number of CACTA superfamily characteristic features. CACTA superfamily transposable elements are DNA transposons directly transposed from DNA to DNA (Feschotte *et al.*, 2002), and almost all CACTA transposable elements harbour 'CACTA' in the outermost region of TIR (Tian, 2006). *Tdv1* also has a CACTA superfamily characteristic TIR (CACTACAA) (Table 2) and a 3 bp (TGG) TSD was observed in the MJY genome (Fig. 5B). The 606 amino acid ORF in *Tdv1* shared high identity with other CACTA superfamily transposable elements [41% identical to TNP2 in *A. majus Tam1* (Nacken *et al.*, 1991), 33% identical to TNP2 in *G. max Tgm9* (Xu *et al.*, 2010), and 32% identical to *En-1* in *Z. mays* (Pereira *et al.*, 1986)]. Further analysis of this translated amino acid sequence by NCBI's CDD (Marchler-Bauer *et al.*, 2011) showed high identity with Transposase_21 (pfam02992). The footprint sequences formed by excision of the transposable element were also found in the MJW genome (Table 3, Fig. 5C), indicating that *Tdv1* is an active CACTA superfamily transposable element. Because no PCR fragments with footprint sequences of *Tdv1* in MJY could be amplified, transposition of *Tdv1* is completely suppressed in MJY.

Anthocyanin and butein synthesis in dahlia

Flavones and butein accumulated in the ray florets of both MJOr and MJY. Thus, to synthesize these compounds, at least one *CHS* other than *DvCHS1* must function in MJY. Indeed, another *CHS*, *DvCHS2*, coding for a 398 amino acid protein, was identified whereas *DvCHS1* coded for a 389 amino acid protein. The nucleotides from the start to the stop codon and the amino acid sequence in *DvCHS1* were 69% and 82% identical, respectively, to those in *DvCHS2*. A phylogenic tree was constructed using the deduced amino acid sequences associated with flavonoid synthesis, including *DvCHS1* and *DvCHS2* (Supplementary Fig. S1 at *JXB* online). *DvCHS1* showed high identity with *GhCHS4* (88%) and *DvCHS2* showed high identity with *GhCHS1* (89%) and *GhCHS3* (84%). *GhCHS1* and *GhCHS3* are associated with anthocyanin and flavonol synthesis in the corolla of gerbera

(Helariutta *et al.*, 1995), and *GhCHS4* is expressed in ray florets and carpels and is a typical CHS that synthesizes naringenin chalcone (Laitinen *et al.*, 2008). CHS generally functions as a homodimer (Ferrer *et al.*, 1999); therefore, amino acid residues associated with the formation of active sites are commonly conserved in plants. Cys164, His303, and Asn336, as CoA-binding sites, Val98, Thr132, Ser133, Met137, Gly163, Thr194, Val196, Thr197, Gly211, Gly216, Ile254, Gly256, Ser338, and Pro375 as active site residues, and Phe215 and Phe265 as gatekeepers are conserved in CHS2 of *Medicago sativa* (Ma *et al.*, 2009). *DvCHS1* and *DvCHS2* possess all these conserved residues. These observations indicate that *DvCHS1* and *DvCHS2* encode functional CHS proteins. Therefore, although whether *DvCHS1* and *DvCHS2* have functional selectivity is still unclear, presumably *DvCHS2* but not *DvCHS1* contributes to synthesis of flavones or butein in MJY, and regulation of anthocyanin and flavone/butein synthesis is different in dahlia.

Redundant copies regulated by DvIVS

Dahlia is an autoallooctoploid (Lawrence, 1929, 1931*b*; Lawrence and Scott-Honcrieff, 1935; Gatt *et al.*, 1998). *DvCHS1* (Supplementary Table S7 at *JXB* online), *DvCHI* (Supplementary Table S8), *DvF3H* (Supplementary Table S9), and *DvDFR* (Supplementary Table S10) mRNA sequences expressed in MJOr and MJY ray florets wer sequenced and the number of different sequences expressed in both lines was determined on the basis of SNPs. Sequences of *DvCHI*, *DvF3H*, and *DvDFR* cDNA were almost the same in MJOr and MJY. However, a drastic difference was detected in the expression sequence of

DvCHS1. At least three different major transcripts were detected in MJOr, but only one transcript was detected in MJY. This difference was confirmed by DGGE analysis (Supplementary Fig. S2 at *JXB* online). The genomic region of *DvCHS1* of MJOr, MJW, and MJY was sequenced (Supplementary Tables S11, S12). Three common sequences were detected among these three lines. Thus, mRNA differences were derived not from genomic differences, but presumably from differences in *DvIVS* transcriptional regulation. Although the expression level of *DvCHS1* was much lower in MJY (Fig. 3C), one type of *DvCHS1* mRNA expressed in MJY was also expressed in MJOr, suggesting that *DvCHS1* expression is not completely regulated by DvIVS. *DvCHS1* can be divided into two groups: the regulation of the first is totally dependent on DvIVS, while the other can also be activated independently of DvIVS. The common sequence belongs to the latter group and the others belong to the former group. This imperfect regulation might be derived from genetic redundancy. Nevertheless, further study is needed in this regard. The manner in which plants with higher polyploidy regulate redundant genes is very interesting.

Finally, a putative model of anthocyanin and butein synthesis in MJOr and MJY was constructed (Fig. 6). In MJOr, DvIVS functions, followed by activation of *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS* and synthesis of anthocyanidin, flavone, and butein. In MJY, because of *Tdv1* insertion, only partial *DvIVS* mRNA is expressed and transcriptional regulation cannot function. Although *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS* are not activated, *DvCHS2* and *DvCHI* are not affected by DvIVS, resulting
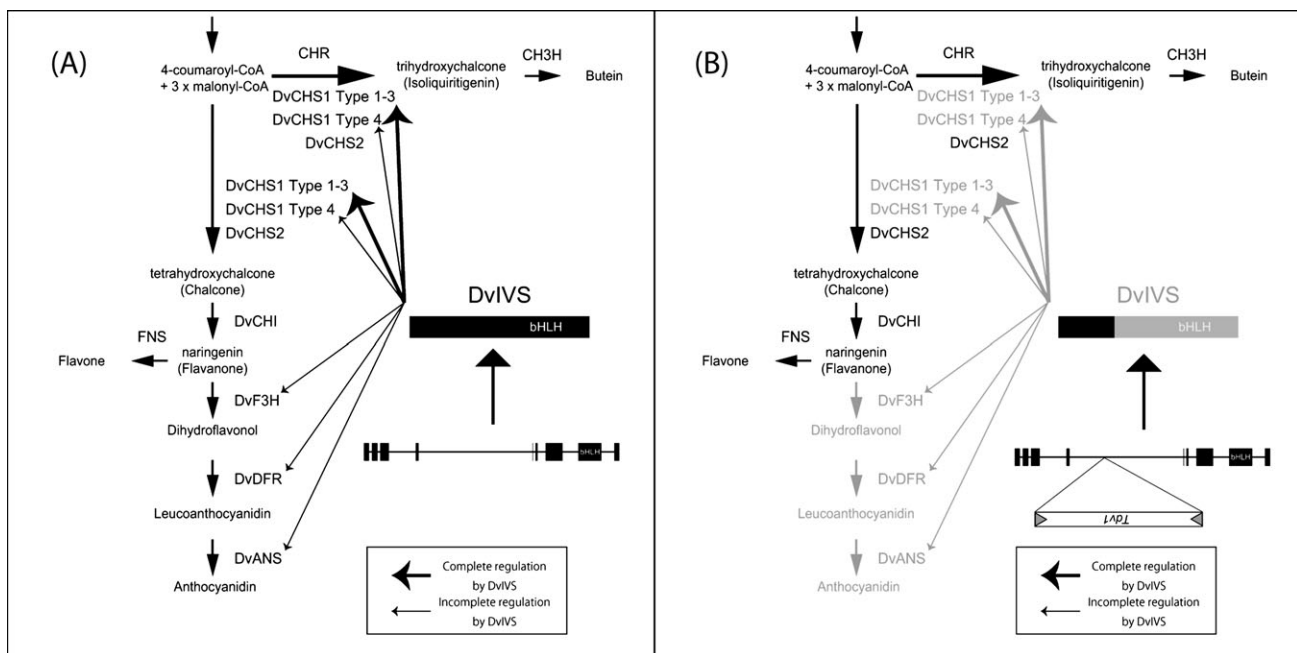

Fig. 6. The putative model of anthocyanin and butein synthesis in MJOr and MJY. For simplicity, the 3′ hydroxylation pathway is abbreviated. (A) The model for MJOr. DvIVS can function, then *DvCHS1, DvF3H, DvDFR,* and *DvANS* are activated, and anthocyanidin, flavone, and butein are synthesized. (B) The model for MJY. Because of *Tdv1* insertion, only truncated *DvIVS* mRNA is expressed and transcriptional regulation cannot function. Although *DvCHS1, DvF3H, DvDFR*, and *DvANS* are not activated, *DvCHS2* and *DvCHI* are not affected by DvIVS, resulting in accumulation of flavone and butein.

in accumulation of flavone and butein. Therefore, MJOr produces orange ray florets on account of the accumulation of anthocyanin and butein, while MJY produces yellow ray florets on account of the accumulation of butein only.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Phylogenetic tree for CHS associated with flavonoid synthesis.

Figure S2. DGGE analysis of *DvCHS1* cDNA from MJOr and MJY.

Table S1. Degenerate primers used for isolating the cDNA fragment.

Table S2. Primers used for RACE.

Table S3. Primers used for semi-quantitative RT-PCR.

Table S4. Primers used for real-time RT-PCR.

Table S5. Primers used for analysing the *DvIVS* genomic region and *Tdv1* region.

Table S6. Primers used for confirming the sequence.

Table S7. SNPs of *DvCHS1* cDNA in MJOr and MJY.

Table S8. SNPs of *DvCHI* cDNA in MJOr and MJY.

Table S9. SNPs of *DvF3H* cDNA in MJOr and MJY.

Table S10. SNPs of *DvDFR* cDNA in MJOr and MJY.

Table S11. SNPs in the exon of the *DvCHS1* genomic region in MJOr, MJY, and MJW.

Table S12. SNPs in the intron of the *DvCHS1* genomic region in MJOr, MJY, and MJW.

Acknowledgements

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