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RESEARCH PAPER

A bHLH transcription factor, \textit{DvIVS}, is involved in regulation of anthocyanin synthesis in dahlia (\textit{Dahlia variabilis})

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Abstract

Dahlias (\textit{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 \textit{chalcone synthase} \textsuperscript{1} (\textit{DvCHS1}), \textit{flavanone 3-hydroxylase} (\textit{DvF3H}), \textit{dihydroflavonol 4-reductase} (\textit{DvDFR}), \textit{anthocyanidin synthase} (\textit{DvANS}), and \textit{DvIVS} encoding a basic helix–loop–helix transcription factor were suppressed, whereas that of \textit{chalcone isomerase} (\textit{DvCHI}) and \textit{DvCHS2}, another \textit{CHS} with 69% nucleotide identity with \textit{DvCHS1}, was not suppressed in the yellow ray florets of MJY. A 5.4 kb CACTA superfamily transposable element, \textit{transposable element of Dahlia variabilis 1} (\textit{Tdv1}), was found in the fourth intron of the \textit{DvIVS} gene of MJW and MJY, and footprints of \textit{Tdv1} were detected in the variegated flowers of MJW. It is shown that only one type of \textit{DvIVS} gene was expressed in MJOr, whereas these plants are likely to have three types of the \textit{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 (\textit{Dahlia variabilis}) are popular ornamental plants cultivated in many countries. They belong to the Asteraceae and are autoallooctoploids with chromosome number 2\textit{n} = 64 (Lawrence, 1929, 1931; Lawrence and Scott-Honcrieff, 1935; Gatt \textit{et al.}, 1998). Because of high polyploidy, dahlias exhibit various inflorescence shapes, colours, and sizes, and \textasciitilde 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 \textit{et al.}, 1955; Harborne \textit{et al.}, 1990; Yamaguchi \textit{et al.}, 1999). In previous studies, four elements were believed to explain the
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-coumaryl-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-coumaryl-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.
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, RF 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 cetyltrimethylammonium bromide method (Murray and Thompson, 1980) and
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*, *DwWDR1*, and *DwFWR2* 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 polymorphism. 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* ‘Matsubirubayashi’ 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 *Kod* 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 5′IVS 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*, *DvCHI*, 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 were used: GGE-F (5’-CCCGGGCGGGCCCGCGATCTTGGA-3’) and GGE-R (5’-CCAGATTTCAAGCACACATTGCTT-3’). After confirming a single band by agarose gel electrophoresis, peridDGGE 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 (DvCHI).
Results

Pigment analysis

Wild-type MJW produces yellow ray florets with orange variegation, while MJO and MJY produce completely orange and completely yellow ray florets, respectively, without any variegation. To clarify the difference in pigments between MJO 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 MJO but not in MJY (Table 1). These data showed that chalcones and flavones are synthesized in both lines, while anthocyanidins are synthesized only in MJO.

Table 1. Aglycones in the petals of MJO and MJY

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

+, Abundant; –, not detected.

To analyse genes associated with anthocyanin synthesis, temporal expression of anthocyanin synthesis pathway genes during ray floret development in MJO and MJY was determined on the basis of SNPs. Based on sequence analysis of transcripts, 17 and 15 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 InWDR1 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 MJO ray florets were divided into five developmental stages based on the degree of colouration and RT-PCR was performed. Although expression levels of DvCHS1 and DvCHI showed no difference, expression levels of DvCHS1, DvF3H, DvDFR, and DvANS were lower in MJY than in MJO 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 MJO, 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 MJO, 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 MJO 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.

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/
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 retials 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; ln, Ipomoea nil; Pfi, Petunia frutescens; Ph, Petunia hybrida; Zm, Zea mays. The accession number of each protein is: DvIVS (ABJ33515), DvDEL (ABJ33516), AmDEL (AAA32653), AtEG3 (NP_176552), AtGL3 (NP_680372), AtTT8 (CAC14865), IndEL (BAE94393), InIVS (BAE94394), PfF3G1 (BAC56998), PhAN1 (AAG25927), PhJAF13 (AAC39455), and ZmN1 (AAAB3841).

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 Anl 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 (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.
Discussion

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

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 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     | CACTACAAGAAA          | 13          | Zea mays                    | Pereira et al. (1986)    |
| Tam1    | CACTACAACAAAAA        | 13          | Antirrhinum majus           | Nacken et al. (1991)     |
| Car1    | CACTACAA              | 8           | Arabidopsis thaliana        | Miura et al. (2001)      |
| Tpn1    | CACTACAAGAAAAATGCCATAGCAAC | 27 | Pharbitis nil (Ipomoea nil) | Inagaki et al. (1994)    |
| Tgm1    | CACTATTAGAAAA         | 13          | Glycine max                 | Vodkin et al. (1983)     |
| Cs1     | CACTATGCCAGAAAAAGCTTA | 20          | Sorghum bicolor             | Chopra et al. (1999)     |
| Pis1    | CACTACGCCAAAAA        | 12          | Psism sativum               | Shirsat (1988)           |
| Ps1     | CACTACAAAAAAA         | 12          | Petunia hybrida             | Snowden and Napoli (1998) |
| Tdc1    | CACTACAGAGAAAACGGAGAGA | 20          | Daucus carota               | Ozeki et al. (1997)      |
| Trn3    | CACTAAGAGGAGAT        | 13          | Oryza sativa                | Motohashi et al. (1996)  |

This table is based on Tian (2006).
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, Anl, and IntVS, 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 Anl (Spelt et al., 2000), IntVS (Park et al., 2007), AtTT8 (Nesi et al., 2000), and GbhHLH1 in Gentiana triflora (Nakatsuka et al., 2008) are encoded within a single exon, while those of JAF13 and ZmInl are encoded within two exons. DvIVS shows high identity with Anl (50%), IntVS (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 Anl gene. These data suggest that DvIVS is an orthologue of AtTT8, Anl, and IntVS, 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 DvCHSI 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 synthesise 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.

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* |
| 2    | 625         | 97                              | 6    | 16  | 24  |
| 3    | 615         | 97                              | 1    | 3   | 16  |

* Footprint sequences were not considered in type 1 of MJW.

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 hAT 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 MJY. 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 DvCHSI must function in MJY. Indeed, another CHS, DvCHS2, coding for a 398 amino acid protein, was identified whereas DvCHSI coded for a 389 amino acid protein. The nucleotides from the start to the stop codon and the amino acid sequence in DvCHSI were 69% and 82% identical, respectively, to those in DvCHS2. A phylogenetic tree was constructed using the deduced amino acid sequences associated with flavonoid synthesis, including DvCHSI and DvCHS2 (Supplementary Fig. S1 at JXB online). DvCHSI showed high identity with GhCHS4 (88%) and DvCHS2 showed high identity with GhCHS1 (89%) and GhCHS3 (84%). GhCHSI and GhCHS3 are associated with anthocyanin and flavonol synthesis in the corolla of gerbera.

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and \( \text{GhCHS4} \) 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 \( \text{Medicago sativa} \) (Ma et al., 2009). \( \text{DvCHS1} \) and \( \text{DvCHS2} \) possess all these conserved residues. These observations indicate that \( \text{DvCHS1} \) and \( \text{DvCHS2} \) encode functional CHS proteins. Therefore, although whether \( \text{DvCHS1} \) and \( \text{DvCHS2} \) have functional selectivity is still unclear, presumably \( \text{DvCHS2} \) but not \( \text{DvCHS1} \) 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 \( \text{DvIVS} \)**

Dahlia is an autoallooctoploid (Lawrence, 1929, 1931a; Lawrence and Scott-Honcrieff, 1935; Gatt et al., 1998). \( \text{DvCHS1} \) (Supplementary Table S7 at \textit{JXB} online), \( \text{DvCHI} \) (Supplementary Table S8), \( \text{DvF3H} \) (Supplementary Table S9), and \( \text{DvDFR} \) (Supplementary Table S10) mRNA sequences expressed in MJOr and MJY ray florets were sequenced and the number of different sequences expressed in both lines was determined on the basis of SNPs. Sequences of \( \text{DvCHI} \), \( \text{DvF3H} \), and \( \text{DvDFR} \) cDNA were almost the same in MJOr and MJY. However, a drastic difference was detected in the expression sequence of \( \text{DvCHS1} \). 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 \textit{JXB} online). The genomic region of \( \text{DvCHS1} \) 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 \( \text{DvIVS} \) transcriptional regulation. Although the expression level of \( \text{DvCHS1} \) was much lower in MJY (Fig. 3C), one type of \( \text{DvCHS1} \) mRNA expressed in MJY was also expressed in MJOr, suggesting that \( \text{DvCHS1} \) expression is not completely regulated by \( \text{DvIVS} \). \( \text{DvCHS1} \) can be divided into two groups: the regulation of the first is totally dependent on \( \text{DvIVS} \), while the other can also be activated independently of \( \text{DvIVS} \). 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, \( \text{DvIVS} \) functions, followed by activation of \( \text{DvCHS1} \), \( \text{DvF3H} \), \( \text{DvDFR} \), and \( \text{DvANS} \) and synthesis of anthocyanidin, flavone, and butein. In MJY, because of \( \text{Tdv1} \) insertion, only partial \( \text{DvIVS} \) mRNA is expressed and transcriptional regulation cannot function. Although \( \text{DvCHS1} \), \( \text{DvF3H} \), \( \text{DvDFR} \), and \( \text{DvANS} \) are not activated, \( \text{DvCHS2} \) and \( \text{DvCHI} \) are not affected by \( \text{DvIVS} \), resulting

![Fig. 6](http://.../JXBonline/supplementary_images/Fig_6.png)

**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. \( \text{DvIVS} \) can function, then \( \text{DvCHS1} \), \( \text{DvF3H} \), \( \text{DvDFR} \), and \( \text{DvANS} \) are activated, and anthocyanidin, flavone, and butein are synthesized. (B) The model for MJY. Because of \( \text{Tdv1} \) insertion, only truncated \( \text{DvIVS} \) mRNA is expressed and transcriptional regulation cannot function. Although \( \text{DvCHS1} \), \( \text{DvF3H} \), \( \text{DvDFR} \), and \( \text{DvANS} \) are not activated, \( \text{DvCHS2} \) and \( \text{DvCHI} \) are not affected by \( \text{DvIVS} \), 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. Phylgenetic 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.

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**References**

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.

Bate-Smith EC, Swain T, Nördstrom CG. 1955. Chemistry and inheritance of flower colour in the Dahlia. *Nature* 176, 1016–1018.

Bomati EK, Austin MB, Bowman ME, Dixon RA, Noel JP. 2005. Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis. *Journal of Biological Chemistry* 280, 30496–30503.

Broerjes C, Ballego JM. 1967. Mutation breeding of *Dahlia variabilis*. *Euphytica* 16, 171–176.

Chopra S, Brendel V, Zhang J, Axtell JD, Peterson T. 1999. Molecular characterization of a mutable pigmentation phenotype and isolation of the first active transposable element from *Sorghum bicolor*. *Proceedings of the National Academy of Sciences, USA* 96, 15330–15335.

Chuck G, Robbins T, Nijjar C, Ralston E, Courtney-Gutterson N, Dooner HK. 1993. Tagging and cloning of a petunia flower color gene with the maize transposable element Activator. *The Plant Cell* 5, 371–378.

De Vetten N, Quattrocchio F, Mol J, Koes R. 1997. The an11 locus controlling flower pigmentation in petunia encodes a novel WD-repet protein conserved in yeasts, plants, and animals. *Genes and Development* 11, 1422–1434.

Fedoroff NV, Furtek DB, Nelson OE. 1984. Cloning of the bronze locus in maize by a simple and generalizable procedure using the transposable controlling element Activator (Ac). *Proceedings of the National Academy of Sciences, USA* 81, 3825–3829.

Ferrer JL, Jez JM, Bowman ME, Dixon RA, Noel JP. 1999. Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. *Nature Structural Biology* 6, 775–784.

Feschotte C, Jiang N, Wessler SR. 2002. Plant transposable elements: where genetics meets genomes. *Nature Reviews Genetics* 3, 329–341.

Fischer D, Stich K, Britsch L, Grisebach H. 1988. Purification and characterization of (+) dihydroflavonol (3-hydroxyflavanone) 4-reductase from flowers of *Dahlia variabilis*. *Archives of Biochemistry and Biophysics* 264, 40–47.

Gatt M, Ding H, Hammett K, Murray B. 1998. Polyploidy and evolution in wild and cultivated *Dahlia* species. *Annales of Botany* 81, 647–656.

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM. 2008. Regulation of the anthocyanin biosynthetic pathway by the TGT1/bHLH/MYb transcriptional complex in Arabidopsis seedlings. *The Plant Journal* 53, 814–827.

Habu Y, Hisatomi Y, Iida S. 1998. Molecular characterization of the mutable flaked allele for flower variegation in the common morning glory. *The Plant Journal* 16, 371–376.

Harborne JB, Greenham J, Eagles J. 1990. Malonylated chalcone glycosides in *Dahlia*. *Phytochemistry* 29, 2899–2900.

Helariutta Y, Elomaa P, Kotilainen M, Griesbach RJ, Schroder J, Teeri TH. 1995. Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in *Gerbera hybrida* (Asteraceae). *Plant Molecular Biology* 28, 47–60.

Inagaki Y, Hisatomi Y, Suzuki T, Kasahara K, Iida S. 1994. Isolation of a Suppressor-mutator/Enhancer-like transposable element, Tpn1, from Japanese morning glory bearing variegated flowers. *The Plant Cell* 6, 375–383.

Koes R, Verweij W, Quattrocchio F. 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends in Plant Science* 10, 236–242.

Laitinen RAE, Ainasoja M, Broholm SK, Teeri TH, Elomaa P. 2008. Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*. *Journal of Experimental Botany* 59, 3691–3703.

Lawrence WJC. 1929. The genetics and cytology of *Dahlia* species. *Journal of Genetics* 21, 125–159.

Lawrence WJC. 1931a. The genetics and cytology of *Dahlia variabilis*. *Journal of Genetics* 24, 257–306.

Lawrence WJC. 1931b. The secondary association of chromosomes. *Cytologia* 2, 352–384.
DvIVS is involved in anthocyanin synthesis in dahlia

Lawrence WJC, Scott-Honcrieff R. 1935. The genetics and chemistry of flower colour in Dahlia: a new theory of specific pigmentation. Journal of Genetics 30, 155–226.

Ludwig SR, Habera LF, Dellaporta SL, Wessler SR. 1989. Lc, a member of the maize R gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. Proceedings of the National Academy of Sciences, USA 86, 7092–7096.

Ma LQ, Pang XB, Shen HY, Pu GB, Wang HH, Lei CY, Wang H, Li GF, Liu BY, Ye HC. 2009. A novel type III polyketide synthase encoded by a three-intron gene from Polygonum cuspidatum. Planta 229, 457–469.

Marchler-Bauer A, Lu S, et al. 2011. CDD: a conserved domain database for the functional annotation of proteins. Nucleic Acids Research 39, D225–D229.

Martin C, Carpenter R, Sommer H, Saedler H, Coen ES. 1985. Molecular analysis of instability in flower pigmentation of Antirrhinum majus, following isolation of the palilda locus by transposon tagging. EMBO Journal 4, 1625–1630.

McClaren M. 2009. Dahlia: history and species. In: McClaren B, ed. Encyclopedia of dahlias. Portland, OR: Timber Press, 161–166.

Miura A, Yonebayashi S, Watanabe K, Toyama T, Watanabe K, Shimada H, Kakutani T. 2006. Isolation and characterization of R2R3-MYB and bHLH transcription factors regulating anthocyanin biosynthesis in gentian flowers. The Plant Journal 49, 411–424.

Nakatsuka T, Haruta KS, Pitaksutheepong C, Abe Y, Kakizaki Y, Yamamoto K, Shimada N, Yamamura S, Nishihara M. 2008. Identification and characterization of R2R3-MYB and bHLH transcription factors regulating anthocyanin biosynthesis in gentian flowers. Plant and Cell Physiology 49, 4321–4325.

Nacken WK, Piotrowiak R, Saedler H, Sommer H. 1991. The molecular and general genetics of a suppressor-mutator/Enhancer and identification of c2006. Isolation and characterization of anthocyanin genes. Plant and Cell Physiology 47, 457–470.

Nakatsuka T, Haruta KS, Pitaksutheepong C, Abe Y, Kakizaki Y, Yamamoto K, Shimada N, Yamamura S, Nishihara M. 2008. Identification and characterization of R2R3-MYB and bHLH transcription factors regulating anthocyanin biosynthesis in gentian flowers. Plant and Cell Physiology 49, 1818–1829.

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L. 2000. The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis silique. The Plant Cell 12, 1863–1878.

Nordström CG, Swain T. 1953. The flavonoid glycosides of Dahlia variabilis. Part I. General introduction. Cyanidin, apigenin, and luteolin glycosides from the variety ‘Dandy’. Journal of the Chemical Society, 2764–2773.

Nordström CG, Swain T. 1956. The flavonoid glycosides of Dahlia variabilis. II. Glycosides of yellow varieties ‘Pius IX’ and ‘Coton’. Archives of Biochemistry and Biophysics 60, 329–344.

Nordström CG, Swain T. 1958. The flavonoid glycosides of Dahlia variabilis. III. Glycosides from white varieties. Archives of Biochemistry and Biophysics 73, 220–223.

Ogata J, Sakamoto T, Yamaguchi MA, Kawanobu S, Yoshitama K. 2001. Isolation and characterization of anthocyanin 5-O-glucosyltransferase from flowers of Dahlia variabilis. Journal of Plant Physiology 158, 709–714.

Ozeki Y, Davies E, Takeda J. 1997. Somatic variation during long term subculturing of plant cells caused by insertion of a transposable element in a phenylalanine ammonia-lyase (PAL) gene. Molecular and General Genetics 254, 407–416.

Park Ki, Choi JD, Hoshino A, Morita Y, Iida S. 2004. An intragenic tandem duplication in a transcriptional regulatory gene for anthocyanin biosynthesis confers pale-colored flowers and seeds with fine spots in Ipomoea tricolor. The Plant Journal 38, 840–849.

Park Ki, Ishikawa N, Morita Y, Choi JD, Hoshino A, Iida S. 2007. A bHLH regulatory gene in the common morning glory, Ipomoea purpurea, controls anthocyanin biosynthesis in flowers, proanthocyanidin and phytomelanin pigmentation in seeds, and seed trichome formation. The Plant Journal 49, 641–654.

Payne CT, Zhang F, Lloyd AM. 2000. GL3 encodes a bHLH protein that regulates trichome development in arabidopsis through interaction with GL1 and TTG1. Genetics 156, 1349–1362.

Pereira A, Cuypers H, Gieri A, Schwarz-Sommer Z, Saedler H. 1996. Molecular analysis of the En/Spm transposable element system of Zea mays. EMBO Journal 5, 835–841.

Quattrocchio F, Wing JF, Leppen HTC, Moi JNM, Koes RE. 1993. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. The Plant Cell 5, 1497–1512.

Quattrocchio F, Wing JF, Van der Woude K, Moi JNM, Koes R. 1998. Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. The Plant Journal 13, 475–488.

Saito N, Ishizuka K, Osawa Y. 1970. Paper-chromatographic identification of flavonoids from a scarlet-flowering dahlia and crystallization of pelargonidin and butein. Botanical Magazine Tokyo 83, 229–232.

Schlangen K, Miosic S, Halbwirth H. 2010a. Allelic variants from Dahlia variabilis encode flavonoid 3′-hydroxylases with functional differences in chalcone 3′-hydroxylase activity. Archives of Biochemistry and Biophysics 494, 40–45.

Schlangen K, Miosic S, Thill J, Halbwirth H. 2010b. Cloning, functional expression, and characterization of a chalcone 3′-hydroxylase from Cosmos sulphureus. Journal of Experimental Botany 61, 3451–3459.

Schlangen K, Miosic S, Topuz F, Muster G, Marosits T, Seitz C, Halbwirth H. 2009. Chalcone 3′-hydroxylase is not a general property of flavonoid 3′-hydroxylase. Plant Science 177, 97–102.
Shirsat AH. 1988. A transposon-like structure in the 5’ flanking sequence of a legumin gene from *Pisum sativum*. *Molecular and General Genetics* **212**, 129–133.

Singh JP, Arora RS, Dohare SR, Sengupta K. 1970. A spontaneous mutant for flower colour and shape in a white flowering dahlia. *Euphytica* **19**, 261–262.

Snowden KC, Napoli CA. 1998. Psl: a novel Spm-like transposable element from *Petunia hybrida*. *The Plant Journal* **14**, 43–54.

Spelt C, Quattrocchio F, Mol JNM, Koes R. 2000. *anthocyanin1* of petunia encodes a basic helix–loop–helix protein that directly activates transcription of structural anthocyanin genes. *The Plant Cell* **12**, 1619–1631.

Spelt C, Quattrocchio F, Mol J, Koes R. 2002. **ANTHOCYANIN1** of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. *The Plant Cell* **14**, 2121–2135.

Suzuki H, Nakayama T, Yonekura-Sakakibara K, Fukui Y, Nakamura N, Yamaguchi MA, Tanaka Y, Kusumi T, Nishino T. 2002. cDNA cloning, heterologous expressions, and functional characterization of malonyl-coenzyme A: anthocyanidin 3-O-glucoside-6′-O-malonyltransferase from dahlia flowers. *Plant Physiology* **130**, 2142–2151.

Tanaka Y, Sasaki N, Ohmiya A. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *The Plant Journal* **54**, 733–749.

Tian PF. 2006. Progress in plant CACTA elements. *Acta Genetica Sinica* **33**, 765–774.

Toledo-Ortiz G, Huq E, Quail PH. 2003. The Arabidopsis basic/helix–loop–helix–loop transcription factor family. *The Plant Cell* **15**, 1749–1770.

Vodkin LO, Rhodes PR, Goldberg RB. 1983. Ca lectin gene insertion has the structural features of a transposable element. *Cell* **34**, 1023–1031.

Wimmer G, Halbwrith H, Wurst F, Forkmann G, Stich K. 1998. Enzymatic hydroxylation of 6′-deoxychalcones with protein preparations from petals of *Dahlia variabilis*. *Phytochemistry* **47**, 1013–1016.

Winkel-Shirley B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiology* **126**, 485–493.

Xu M, Brar HK, Grosic S, Palmer RG, Bhattacharyya MK. 2010. Excision of an active CACTA-like transposable element from *DFR2* causes variegated flowers in soybean (*Glycine max* (L.) Merr.). *Genetics* **184**, 53–63.

Yamaguchi MA, Oshida N, Nakayama M, Koshioka M, Yamaguchi Y, Ino I. 1999. Anthocyanidin 3-glucoside malonyltransferase from *Dahlia variabilis*. *Phytochemistry* **52**, 15–18.

Yamasaki H, Uefuji H, Sakihama Y. 1996. Bleaching of the red anthocyanin induced by superoxide radical. *Archives of Biochemistry and Biophysics* **332**, 183–186.

Zabala G, Vodkin L. 2008. A putative autonomous 20.5 kb-CACTA transposon insertion in an *F3'H* allele identifies a new CACTA transposon subfamily in *Glycine max*. *BMC Plant Biology* **8**, 124.

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A. 2003. A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. *Development* **130**, 4859–4869.