Localized Repression of Two bHLH Genes is Involved in the Formation of White Margins and White Abaxial Surfaces in Carnation Petals by Inducing the Absence of Anthocyanin Synthesis

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Flower color patterns are attractive traits of floricultural plants. However, the mechanisms underlying these traits remain mostly unknown. Carnation (Dianthus caryophyllus L.) and interspecific hybrids thereof exhibit many flower color patterns involving white margins on reddish petals, as observed in the cultivar ‘Minerva’. Flowers with white margins also have white abaxial surfaces. We studied the factors regulating the formation of white margins and a white abaxial surface in flowers through analyses of pigments and related gene expression. HPLC analysis revealed an absence of anthocyanins in white margins, although the accumulation of other flavonoid pigments (flavonols) was almost identical between the dark-red central and white marginal regions of petals. RNA-seq analyses of the dark red central regions and white marginal regions of ‘Minerva’ petals resulted in the extraction of 18 genes related to anthocyanin biosynthesis and transportation, including some transcription factors, as candidate regulatory genes for the formation of white margins. Further analysis of the expression of these genes by real-time RT-PCR and a comparison of two white-margin-flowered cultivars and three red-unicolor-flowered cultivars indicated that the expressions of two bHLH transcription factor genes and seven structural genes were positively correlated with anthocyanin accumulation. Although DcbHLH1, which is a homolog of JAF13 in Petunia × hybrida, was expressed in both flower color groups, DcbHLH2, a homolog of AN1, was expressed only in white-marginal-flowered cultivars. Moreover, in the petals of ‘Minerva’, the expression of those nine genes was repressed in the abaxial epidermal layer without red pigmentation conferred by anthocyanins. Therefore, we could postulate that the localized repression of both bHLH genes is involved in the formation of white margins in carnation petals by inducing the absence of anthocyanin synthesis and that the white abaxial surface of the petals may result from similar regulatory mechanisms. In particular, DcbHLH2 could act as a key gene because of its restricted expression only in cultivars with white-margined flowers.

Key Words: laser microdissection, MBW complex, picotee, proanthocyanidin.

Introduction

Flower color patterns consisting of multiple colors are attractive traits and important commercial characteristics of floricultural plants. In many angiosperms, large variations in flower color patterns are observed. Because of not only the beautiful appearance conferred by flower color patterns, but also their clear visual perception, they are of great interest for clarifying the mechanisms of the formation. Flower color patterns are a result of the spatially and temporally restricted accumulation of pigments, especially cyanic color patterns produced by anthocyanins, which are flavonoids with a range of red to blue colors. The anthocyanin bio-
synthesis pathway is well characterized, and most of the genes encoding biosynthesis enzymes in this pathway have been identified and isolated from many plant species (Tanaka et al., 2008). The expression of these structural genes is regulated by transcription factors that have mainly been identified in model species, including maize (Zea mays), petunia (Petunia × hybrida), snapdragon (Antirrhinum majus), and Arabidopsis thaliana (Albert et al., 2011; Carey et al., 2004; Cone et al., 1986; de Vetten et al., 1997; Goodrich et al., 1992; Ludwig et al., 1989; Paz-Ares et al., 1986, 1987; Quattrocchio et al., 1998, 1999; Schwinn et al., 2006; Spelt et al., 2000, 2002; Walker et al., 1999). Transcriptional activation complexes composed of R2R3-MYB transcription factors, basic helix-loop-helix (bHLH) transcription factors, and WD40 (WD repeat: WDR) proteins (referred to as MBW complexes) primarily regulate the expression of anthocyanin biosynthesis genes (Hichri et al., 2011; Xu et al., 2015). In Arabidopsis, MBW complexes mainly activate late biosynthetic genes (LBGs), such as dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), flavonoid 3’-hydroxylase (F3’H), and cytosolic UDP-sugar dependent glycosyltransferase (UGTs), while early biosynthetic genes (EBGs), including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3’-hydroxylase (F3’H), and upstream genes involved in phenylpropanoid biosynthesis (phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL)), show different expression patterns determined by different regulatory mechanisms (Gonzalez et al., 2008). The early and late divisions of the flavonoid pathway differ among plant species (Afrin et al., 2014; Gonzalez et al., 2008).

The flower color patterns resulting from the restricted accumulation of anthocyanins are variable and may include spots, stripes, variegation, vein-specific pigmentation (venation), bicolors (including picotee) and different pigmentation on the reverse side of the petals. The mechanisms underlying the restricted accumulation of anthocyanins have been characterized in a few species. There are some reports demonstrating that R2R3-MYB transcription factors other than MYBs controlling full coloration of the entire petal surface regulate spot and venation formation in petunia, snapdragon and some lilies (Albert et al., 2011; Schwinn et al., 2006; Shang et al., 2011; Yamagishi, 2013; Yamagishi et al., 2010, 2018). Spots or stripes are also caused by transposable elements through their insertion into anthocyanin biosynthesis genes (Fujino et al., 2011; Iida et al., 2004; Itoh et al., 2002; Ohno et al., 2011a; van Houwelingen et al., 1999). The bicolor phenotypes of petunia, which are referred to as star or picotee patterns and involve white margins and pigmented centers, and those of dahlia (Dahlia variabilis), in which the basal region is colored and the petal tips are white, are caused by the post-transcriptional gene silencing (PTGS) of CHSs, in white areas (Koseki et al., 2005; Morita et al., 2012; Ohno et al., 2011b). Another type of picotee in petunia involving a pigmented margin and white center is caused by an increase in the expression of flavonol synthase (FLS) in the white center, leading to high flavonol accumulation and lower anthocyanin accumulation due to substrate competition (Saito et al., 2006). However, the mechanisms underlying the various other types of flower color patterns are mostly unknown.

The genus Dianthus comprises many important floricultural species, including the representative species carnation (Dianthus caryophyllus L.). Carnation has a large number of varieties with different flower colors that are attractive in the marketplace. Although some exceptional varieties of carnation with pale-green or pale-yellow flower color can accumulate small amounts of chlorophylls and carotenoids in petals (Iijima et al., 2020; Ohmiya et al., 2014), the majority of carnation varieties mainly accumulate flavonoid pigments, and their flower color phenotypes are genetically regulated by genes related to flavonoid biosynthesis (Geissman and Mehluquist, 1947). Most of these genes and those involved in the vacuolar transport of flavonoids have been identified and have been shown to play a dominant role in flower color variations (Dedio et al., 1995; Itoh et al., 2002; Mato et al., 2000, 2001; Miyahara et al., 2018; Momose et al., 2013a, b; Nishizaki et al., 2011; Okamura et al., 2013; Sasaki et al., 2012, 2013). The carnation genome project has provided considerable information on the genes involved in anthocyanin and flavonoid synthetic pathways (Yagi et al., 2014), and their detailed structures and estimated functions have been discussed by Ozeki et al. (2020).

Carnation includes not only a large number of varieties with a solid (unicolor) flower color, but also many varieties with flower color patterns with multiple colors. Nakayama (2020) divided the flower color patterns of carnations and wild species in the genus Dianthus into 10 types. The ‘Minerva’ cultivar (Fig. 1A) shows a typical phenotype of the “marginal picotee (clearer border)” type described by Nakayama (2020), with white margins and a white abaxial petal surface (Supplemental Fig. S1). Solid white color phenotypes in carnation flowers have been reported to be due to three possible mechanisms: 1) disruption of the A gene responsible for DFR activity (Itoh et al., 2002; Stich et al., 1992), 2) deficiency of F3H expression (Mato et al., 2000), or 3) repression of a bHLH gene resulting in reduced expression of LBGs in the anthocyanin synthetic pathway (Totsuka et al., 2018). However, the mechanism by which the white margins of petals form remains to be elucidated. We compared the transcription of genes related to anthocyanin biosynthesis and transportation between the colored central and white marginal regions of petals in ‘Minerva’ and performed a detailed expression analysis of candidate regulatory
genes between white-margin-flowered and unicolored-red-flowered cultivars. Moreover, the expression of the candidate regulatory genes in three different cell layers of petals was elucidated to investigate the mechanism responsible for the white abaxial surface of the petals. We showed that two bHLHs play a crucial role in these flower color patterns.

Materials and Methods

Plant materials

Two cultivars of Dianthus with white-margined flowers ['Minerva' (Fig. 1A) and 'Black Jack'] and three cultivars with unicolored red flowers ['Anney', ‘Berg’, and ‘Spinel’] were used. ‘Black Jack’ is a hybrid between carnation (D. caryophyllus L.) and several wild species of Dianthus, and the other four cultivars are carnation varieties. Rooted cuttings of ‘Minerva’ were a gift from Kaneko Seeds Co., Ltd. (Maebashi, Japan), and those of ‘Black Jack’, ‘Anney’, ‘Berg’, and ‘Spinel’ were purchased from Miyoshi Group & Co., Ltd. (Tokyo, Japan). Plants were grown in a greenhouse at Chiba University (Matsudo, Japan).

The developmental stages of the petals were defined as follows: stage 1 (S1): in all cultivars, petals are in unopened flower buds without accumulation of anthocyanins; stage 2 (S2): petals are in unopened buds, and the accumulation of anthocyanins conferring red coloration is initiated in ‘Minerva’ and ‘Black Jack’; stage 3 (S3): petals are in unopened buds and become red in ‘Minerva’ and ‘Black Jack’; stage 4 (S4): petals are in unopened buds and become dark red in ‘Minerva’ and ‘Black Jack’; while the other cultivars show no pigmentation (petal length is approximately 1 cm except in ‘Black Jack’, which is 7 mm); stage 5 (S5): petals are in unopened buds, anthocyanin accumulation conferring red coloration is initiated in ‘Anney’, ‘Berg’, and ‘Spinel’; stage 6 (S6): petals immediately after the initiation of flower bud opening at the top (petal length is approximately 3 cm except in ‘Black Jack’, which is 2 cm). Sampled petals were immediately frozen in liquid nitrogen and stored at −80°C until use.

Observation of petal transverse sections

Fully opened fresh petals of each cultivar were sliced transversely with a razor blade. The sections were placed on a glass slide for microscopic observations, a drop of water was added, and the samples were covered with a cover glass. The sections were observed using a Shimadzu BA210E1080 biological light microscope (SHIMADZU RIKA CORPORATION, Tokyo, Japan) at 40 × magnification.

HPLC analysis

Fresh, fully opened petals of each cultivar were divided into central and marginal regions with a razor blade. Samples of central regions were collected from the middle of the red-colored area, and those of marginal regions were collected as approximately 1 mm wide petal peripheries. A sample (25 mg or 50 mg) of each region was frozen and crushed in liquid nitrogen and immersed in 5% HOAc-H2O (1 mL) in a microtube. The mixture was then centrifuged at 4°C at 15,000 rpm for 15 min, and the supernatant was used for analysis. Analytical HPLC was performed on an LC 10A system (Shimadzu Corporation, Kyoto, Japan) using a Waters C18 column (5 μm, 4.6 × 250 mm) at 40°C with a flow rate of 1 mL·min⁻¹, with monitoring at 530 nm for anthocyanins and 350 nm for other flavonoids, including flavonols. The solvent was applied in a linear gradient of 20 to 85% solvent B (1.5% H3PO4, 20% HOAc, 25% MeCN in H2O) mixed with solvent A (1.5% H3PO4 in H2O) over 40 min. To examine anthocyanidin accumulation in each cultivar, extracts containing 5% hydrochloric acid in 50% methanol were heated at 100°C for 1 h in a dry bath and were then analyzed by HPLC under the above conditions. Three or more biological replications for each cultivar were included in the analysis to confirm that there was no significant difference in the chromatogram patterns of a given region in a given cultivar.

RNA-seq analysis of the central and marginal petal regions of ‘Minerva’

The central and marginal regions of ‘Minerva’ S4 petals were subjected to total RNA isolation with a NucleoSpin® RNA kit (Takara Bio Inc., Kusatsu,
RNA-seq library construction was performed with a TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, CA, USA) and sequenced on an Illumina HiSeq 2500 System (Illumina) to obtain paired-end reads (PE100). These sequence data were registered under DDBJ DRA accession number DRA011909. De novo assembly was performed with Trinity and abundance estimation with RSEM. The fragments per kilobase of exon per million reads mapped (FPKM) values of the genes related to the accumulation of anthocyanins and other flavonoids were compared. The genes with fold-change (FC) values higher than 5 or lower than −5, which indicated 5-fold differences in FPKM values between the central and marginal regions, were selected for subsequent qPCR analysis as candidate regulatory genes. Gene Ontology (GO) analysis was performed using the BlastX software tool to compare the six-frame conceptual translation products of a nucleotide query sequence (both strands) against the NCBI protein sequence database (go_v20150407). Sequence identities were determined at the nucleotide level with registered sequences in the Carnation DB (Yagi et al., 2014) and at the amino acid level with the proteins showing GO analysis hits by using the Clustal W method with a gap open penalty = 10.

Sequence analysis of two bHLH cDNAs

Total RNA was extracted from whole petals without the uncolored base parts of all five cultivars using Sepasol®-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) and purified with High-Salt Solution for Precipitation (Takara Bio). The extracted total RNA was subjected to reverse transcription with an oligo dT primer using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). The RT-PCR amplification of full-length DcbHLH1 and DcbHLH2 was performed using primers (Supplemental Table S1) designed for each contig sequence (c28997_g1 and c17941_g1) resulting from de novo RNA-seq assembly. DcbHLH1 was amplified in all five cultivars, and DcbHLH2 was amplified in two white-margin-flowered cultivars. The PCR products were cloned with the pTAC-1 vector and the DynaExpress TA PCR Cloning Kit (BioDynamics Laboratory Inc., Tokyo, Japan). All sequence analyses were performed using a BigDye® Terminator v 3.1 Cycle Sequencing Kit and a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). Each gene was sequenced in at least five clones for each cultivar.

Quantification of gene expression [qPCR]

The quantitation of candidate gene transcripts was performed by real-time RT-PCR. Whole petals in S1 to S6 and the central and marginal regions of petals in S4 and S6 from all cultivars were used. Total RNA was extracted with Sepasol®-RNA I Super G (Nacalai Tesque) and reverse transcribed with ReverTra Ace (Toyobo) using an oligo dT primer. Real-time RT-PCR was performed with a KAPA SYBR Fast qPCR Kit (NIPPNON Genetics Co., Ltd. Tokyo, Japan) using a LightCycler 96 system (Roche Diagnostics K.K., Tokyo, Japan). Real-time RT-PCR was performed as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 3 sec. Single-target product amplification was checked via melting curve analysis. Three biological replications were performed. DcActin was used as an internal standard. The sequences of the primers are given in Supplemental Table S2. All data represent the average ± SE of three replicates.

Laser microdissection and quantification of gene expression in each petal cell layer

Red central regions of ‘Minerva’ petals at S4 were cut out and fixed with fresh ethanol:acetic acid (3:1, v/v) on ice with vacuum deaeration and then at 4°C without vacuum deaeration overnight. The materials were dehydrated twice using 75% ethanol with incubation on ice for 1 h and were then kept at −28°C until freeze embedding. Before embedding, solution replacement was performed as follows: 50% ethanol on ice for 30 min, 25% ethanol on ice for 30 min, pure water on ice for 45 min, 20% sucrose solution on ice for 30 min, 20% sucrose solution:super cryoembedding medium (SCEM) (Leica Microsystems GmbH, Wetzlar, Germany) = 1:1 on ice for 30 min, SCEM on ice for 30 min. Then, the materials were embedded in SCEM in a stainless-steel container. The container was placed in hexane cooled with dry ice for 30 sec until SCEM was completely frozen. The SCEM blocks were kept at −80°C until sections were obtained. Eight μm transverse sections of embedded petals were obtained via Kawamoto’s film method (Kawamoto, 2003). Adhesive films for laser microdissection (LMD film; Leica Microsystems GmbH) and CryoStar™ NX70 (Thermo Fisher Scientific, Waltham, MA, USA) were used. The sections were freeze-dried in CryoStar™ NX70 for 3 h. Each petal cell layer in the dried sections was collected via LMD7 (Leica Microsystems GmbH) (Supplemental Fig. S2). Sampling of the inner layers was performed avoiding vascular bundles. An RNeasy micro kit (Qiagen, Hilden, Germany) was used for RNA extraction from the collected cell layer samples according to the manufacturer’s instructions. Reverse transcription was performed using the Ovation® RNA-seq system V2 (NuGEN Technologies Inc.) followed by the quantification of gene expression as described above.

Results

Accumulation of pigments in the central or marginal region of petals

Transverse sections of petals from the white-marginated cultivars ‘Minerva’ and ‘Black Jack’ showed red pigmentation in the central region of adaxial epidermal cells, but not in the marginal region or abaxial epi-
dermal cells (Fig. 1C). The petals of the other three cultivars showed unicolored red pigmentation in both adaxial and abaxial epidermal cells (Fig. 1D). The inner layer (mesophyll cells) was not pigmented in any of the cultivars.

HPLC analysis revealed that the marginal region of the petals of ‘Minerva’ and ‘Black Jack’ showed little anthocyanin accumulation, whereas the central region accumulated several anthocyanins (Fig. 2; Supplemental Fig. S3). In contrast, other flavonoids (mainly flavonols) detected at 350 nm accumulated in the marginal region, and their levels were higher than those in the central region (Fig. 2; Supplemental Fig. S3). The other three cultivars with unicolored red flowers accumulated anthocyanins and other flavonoids in both the central and marginal regions (Fig. 2; Supplemental Fig. S3). The amounts of these compounds were higher in the marginal regions than in the central region. ‘Minerva’, ‘Black Jack’, and ‘Berg’ accumulated only cyanidin-based anthocyanins, whereas ‘Anney’ and ‘Spinel’ accumulated only pelargonidin-based anthocyanins (data not shown).

Comparative analysis of gene expression at different flower stages and between central and marginal regions of petals

A large number of genes showing differential expression between the central and marginal regions of ‘Minerva’ petals are listed in the RNA-seq results. Some of the identified genes were reported previously to be involved in the biosynthesis and transportation of flavonoids. We chose 18 candidate regulatory genes potentially related to the formation of white-margined flowers: two F3H (DcF3H1 and DcF3H2), DcDFR, DcANS, DcF3'H, DcPAL, DcC4H, Dc4CL, acyl-glucose:anthocyanin 5-O-glucosyltransferase-like (DcAA5GT-like), DcFLS, glutathione S-transferase (DcGST), multidrug toxic compound extrusion (MATE) transporter gene (DcMATE), autohibited H^+-ATPase isoform 10 (DcAHA10), and transcription factor genes, three bHLH genes (DcbHLH1, DcbHLH2, and DcbHLH3) and two MYB genes (DcMYB1 and DcMYB2) (Table 1). Although DcF3H1, DcMYB1, and DcHLH3 showed positive FC values, which indicated higher expression in the white marginal region than in the dark-red central region, other genes showed negative FC values, indicating higher expression in the central region (Table 1). Most of those 18 genes, except for DcAA5GT-like and DcMYB1, corresponded to sequences registered in the Carnation DB (Yagi et al., 2014) and the DB accession numbers are shown in Table 1. The contigs of DcAA5GT-like, DcPAL, DcC4H, DcMATE, DcMYB1, and DcHLH3 were partial sequences for each cDNA, but other contigs contained the full-length cDNA. The length of the nucleotide sequence of DcAA5GT-like was approximately half of the registered sequence of DcAA5GT (Accession No.: AB507446, Matsuba et al., 2010) and showed 66% homology with the last part of DcAA5GT. The nucleotide sequence of DcMYB1 was 67% homologous with that of AtMYB12 (At2G47460, Accession No.: AY519580, Mehrtens et al., 2005). The nucleotide identity of each contig with the corresponding gene in the Carnation DB and the amino acid identity with the best hit gene by BlastX are shown in Table 1. No CHS and CHI were listed as differentially expressed genes because their absolute values of FC were less than 3.

Sequencing analysis of two of three bHLH genes,
Table 1. Candidate regulatory genes for the formation of white-margined flowers extracted from the RNA-seq results for marginal and central petal regions of 'Minerva'.

| Contig     | gene       | Carnation DB | Fold-change | Best BLASTX hit against TAIR Arabidopsis and UniProtKB database | cDNA partial/full | Sequence identity nucleotide | Sequence identity amino acid | Previous reports |
|------------|------------|--------------|-------------|-----------------------------------------------------------------|-------------------|-------------------------------|-----------------------------|------------------|
| c24418_g1_i1 | DcGST     | Dca57804.1   | -118.6      | TAIR|locus:2097730—symbol:GSTF11 AT3G03190                            | full             | 97                            | 50             | DcGSTF2 (Sasaki et al., 2012) |
| c22463_g1_i1 | DcFLS     | Dca60988.1   | -94.2       | TAIR|locus:2159542—symbol:FLS1 AT5G08640                            | full             | 100                           | 47             | DcFLS2 (Ozeki et al., 2020)  |
| c24384_g6_i8 | DcAA5GT-like | —           | -82.5       | UNIPROTKB|E3W9M2—symbol:AA5GT Cyanidin 3-O-glucoside 5-O-glucosyltransferase | partial          | —                             | —              | —                             |
| c29063_g1_i1 | DcAHA10   | Dca60531.1   | -72.0       | TAIR|locus:2020372—symbol:AHA10 AT1G17260                            | full             | 98                            | 78             | —                             |
| c26290_g1_i2 | DcF3′H    | Dca61873.1   | -51.9       | TAIR|locus:2142878—symbol:TT7 AT5G07990                            | full             | 98                            | 64             | DcF3′H (Ozeki et al., 2020)  |
| c25626_g1_i5 | DcMYB2    | Dca55168.1   | -39.8       | TAIR|locus:2009452—symbol:MYB3 AT1G2640                            | full             | 99                            | 36             | —                             |
| c26725_g1_i1 | DcANS     | Dca23371.1   | -38.0       | TAIR|locus:2127218—symbol:LDOX leucoanthocyanidid dioxygenase AT4G22880 | full             | 98                            | 75             | DcANS (Ozeki et al., 2020)  |
| c29068_g1_i1 | DcPAL     | Dca13751.1   | -25.2       | TAIR|locus:2057981—symbol:PAL1 AT2G37040                           | partial          | 99                            | 85             | DcPal2 (Ozeki et al., 2020)  |
| c17941_g1_i2 | DcbHLH2   | Dca38761.1   | -22.3       | TAIR|locus:2118524—symbol:TT8 AT4G09820                           | full             | 98                            | 50             | —                             |
| c23506_g1_i1 | DcDFR     | Dca3242.1    | -18.2       | TAIR|locus:2165427—symbol:DFR AT5G42800                            | full             | 98                            | 68             | DcDFR (Ozeki et al., 2020)  |
| c54854_g1_i1 | Dc4CL     | Dca34373.1   | -17.2       | TAIR|locus:2017602—symbol:4CL1 AT1G51680                            | full             | 99                            | 62             | Dc4CL (Ozeki et al., 2020)  |
| c17333_g2_i1 | DcC4H     | Dca12289.1   | -15.9       | TAIR|locus:2064402—symbol:C4H AT2G30490                            | partial          | 100                           | 79             | DcC4H2 (Ozeki et al., 2020) |
| c7613_g1_i1  | DcbHLH3   | Dca7687.1    | -14.2       | TAIR|locus:2077725—symbol:TT12 AT3G59030                            | full             | 99                            | 72             | —                             |
| c21667_g1_i2 | DcDFR     | Dca38489.1   | -9.9        | TAIR|locus:2081008—symbol:F3H AT3G12540                            | full             | 98                            | 61             | DcF3H2 (Ozeki et al., 2020) |
| c29897_g1_i5 | DcbHLH1   | Dca31716.1   | -8.3        | TAIR|locus:2026629—symbol:ELG3 AT1G63530                            | full             | 98                            | 39             | bHLH (Totsuka et al., 2018)  |
| c23837_g1_i1 | DcF3′H    | Dca38311.1   | 10.5        | TAIR|locus:2081008—symbol:F3H AT3G12540                            | full             | 97                            | 63             | DcF3H3 (Ozeki et al., 2020) |
| c47550_g1_i1 | DcMATE    | —           | 13.6        | TAIR|locus:2062040—symbol:MYB12 AT2G74460                           | partial          | —                             | 44             | —                             |
| c20512_g1_i2 | DcbHLH3   | Dca15903.1   | 16.8        | UNIPROTKB|Q94872—symbol:Plw-OSB2 R-type basic helix-loop-helix protein | partial          | 69                            | 15             | —                             |

a GST: glutathione S-transferase, FLS: flavonol synthase, AA5GT: acyl-glucoseanthocyanin 5-O-glucosyltransferase, AHA10: Autoinhibited H+-ATPase Isoform 10, F3′H: flavonoid 3′-hydroxylase, ANS: anthocyanidin synthase, PAL: phenylalanine ammonia-lyase, bHLH: basic helix-loop-helix transcription factor, DFR: dihydroflavonol 4-reductase, 4CL: 4-coumaroyl CoA ligase, C4H: cinnamate 4-hydroxylase, MATE: multidrug toxic compound extrusion transporter, F3H: flavonone 3-hydroxylase.

b Fold-change values were calculated in fragments per kilobase of exon per million reads mapped (FPKM). Positive values indicate FPKM_marginal region/FPKM_central region, and negative values indicate (−1) × FPKM_central region/FPKM_marginal region.

c This column indicates whether each contig contains a full-length or partial cDNA.

d Sequence identity at the nucleotide level with registered sequences in the Carnation DB determined via the Clustal W method with a gap open penalty = 10.

e Sequence identity at the amino acid level with proteins receiving GO analysis hits determined via the Clustal W method with a gap open penalty = 10.
**DcbHLH1** and **DcbHLH2**, showed that the lengths of the coding region were 1,794 bp and 2,013 bp, respectively. All cultivars had **DcbHLH1** with two insertions (72 bp and 3 bp) relative to Dca31716.1 in the Carnation DB (Accession No.: LC626589, LC626590, LC626591, LC626592, LC626593). The sequence encoded a full-length protein in all cultivars. Eighteen single-nucleotide polymorphisms (SNPs) were detected between the white-margin-flowered and red-unicolor-flowered cultivars, and six of them were accompanied by amino acid substitutions. The **DcbHLH1** mRNA sequence isolated from ‘Minerva’ (Accession No.: LC626594, LC626595) and ‘Black Jack’ (Accession No.: LC626596) was longer in the 5’ direction than Dca38761.1, and this region encoded a bHLH-MYC_N domain (PF14215.5).

Red pigmentation in petals, resulting from the accumulation of anthocyanins, was initiated in an earlier developmental stage in the white-margin-flowered cultivar ‘Minerva’, than in the unicolor-flowered cultivar ‘Berg’ (Fig. 3A). The accumulation of anthocyanins in petals was initiated in S2, and the petals were completely pigmented in S4 in ‘Minerva’; in contrast, pigmentation was initiated in S5 in ‘Berg’. A similar tendency was observed in another white-margin-flowered cultivar ‘Black Jack’ and other unicolor-flowered cultivars ‘Anney’ and ‘Spinel’. Quantitative analysis of the expression of candidate regulatory genes in each stage revealed nine genes for which expression clearly increased in accord with increasing pigmentation. These genes included **DcbHLH1**, **DcF3H1**, **DcF3H2**, **DcFLS**, **DcDFR**, **DcAN5**, **DcGST**, **DcMAT1**, and **DcAHA10** in all cultivars, **DcF3’H** in three cultivars that accumulated cyanidin-based anthocyanins, and **DcbHLH2** in two cultivars with white-margined flowers, ‘Minerva’ and ‘Black Jack’ (Fig. 3). **DcF3H1** and **DcF3H2** were expressed only in ‘Minerva’ and ‘Black Jack’, and **DcAA5GT-like** was expressed only in three cultivars with cyanidin-based anthocyanins (Fig. 3).

Nine genes for which expression was positively correlated with pigmentation in each stage also showed expression patterns correlated with pigmentation in the central and marginal regions of the petals in the white-margin-flowered cultivars ‘Minerva’ and ‘Black Jack’ at S4 (Fig. 4 A–I) and several genes in ‘Black Jack’ in S6 (Fig. 4 A‘–I‘). However, the expression pattern was reversed, with higher expression being found in the marginal region of petals, in the three unicolor-flowered cultivars in S6, when their coloration was complete (Fig. 4 A‘–I‘). A similar tendency was observed for **DcAA5GT-like** (Fig. 4 J, J‘). **DcC4H** was expressed at higher levels in the central region than in the marginal region in ‘Black Jack’, but not in ‘Minerva’ at S4 (Supplemental Fig. S4). The expressions of **DcMYB2**, **DcPML** and **Dc4CL** were higher in the central region of the petals in ‘Minerva’ and ‘Black Jack’, but were also higher in the central region in some unicolor-flowered cultivars (Supplemental Fig. S4). In addition, **DcMYB1** and **DcF3H1** showed a negative correlation with pigmentation in the central and marginal regions of the petals in white-margin-flowered cultivars (Fig. 4 K, K‘, L, L‘). **DcbHLH3** and **DcF3H2** also showed a negative correlation in ‘Black Jack’ at S4, but the correlation was not shown in ‘Minerva’ (Supplemental Fig. S4).

Gene expression analysis in different petal layers

The expressions of 12 genes that are considered to regulate white margins were examined in the adaxial epidermal, inner and abaxial epidermal layers of ‘Minerva’ petals. **DcbHLH2**, **DcFLS**, **DcDFR**, **DcAN5**, **DcF3’H**, **DcGST**, **DcMAT1**, **DcAHA10**, and **DcAA5GT-like** were expressed at significantly higher levels in the adaxial epidermal layer than in the inner or abaxial epidermal layer (Fig. 5). The expression of **DcbHLH1** was also highest in the adaxial epidermal layer, while its expression level was approximately halved in the abaxial layer (Fig. 5). The expression levels of **DcMYB1** and **DcF3H1**, which showed a negative correlation with coloration between the central and marginal regions of the petals in white-margin-flowered cultivars (Fig. 4), were higher in the abaxial epidermal layer than in the adaxial layer (Fig. 5).

Discussion

**White margins of petals show little anthocyanin accumulation**

Observation of transverse sections of petals and HPLC analysis revealed that white margins resulted from a very low level of anthocyanin accumulation in the marginal region of epidermal cell layers (Figs. 1 and 2). The accumulation of other flavonoids detected at 350 nm, especially flavonols, was higher in the marginal regions of the petals in ‘Minerva’ and ‘Black Jack’, as well as the red-unicolored cultivars (Fig. 2; Supplemental Fig. S3), suggesting that this accumulation tendency was not involved in the formation of white margins. The accumulation of anthocyanins in the marginal regions in the petals of unicolored cultivars was also higher than that in the central region (Fig. 2; Supplemental Fig. S3). The marginal regions contained a higher proportion of epidermal cells, which may allow the accumulation of flavonoids, potentially resulting in a higher flavonoid content per unit fresh weight. Therefore, we can postulate that the formation of white margins in the petals of these cultivars could be caused by a very low level of anthocyanin accumulation resulting from the regulation of anthocyanin biosynthesis, transportation or degradation.

**Genes regulating the accumulation of anthocyanins in carnation petals**

Some genes involved in anthocyanin biosynthesis are activated by MBW transcriptional activation complexes, consisting of R2R3-MYB, bHLH and WDR
proteins, and genes encoding these transcription factors have been identified in many plant species, along with the structural genes they regulate (Baudry et al., 2004; Falcone Ferreyra et al., 2012; Hichri et al., 2011; Koes et al., 2005; Ramsay and Glover, 2005; Xu et al., 2015). In this study, the expression of two bHLH genes, DcbHLH1 and DcbHLH2, was positively correlated with anthocyanin accumulation in petals in each developmental stage (Fig. 3). DcbHLH1 and DcbHLH2 are orthologs of EGL3 and TT8 in A. thaliana (Bernhardt et al., 2003; Nesi et al., 2000), JAF13 and AN1 in P. ×hybrida (Quattrocchio et al., 1998; Spelt et al., 2000, 2002) and Delila and Incolorata I (Mutabilis) in snapdragon (A. majus L.) (Albert et al., 2020; Martin and Gerats, 1993; Martin et al., 1991; Schwinn et al., 2006). Among carnation cultivars, DcbHLH1 (Dca31716.1) has been reported to be a regulator of anthocyanin synthesis in red-flowered ‘4-94-1 MR’,

Fig. 3. Developmental stages of petals (A) and relative expression of candidate regulatory genes for anthocyanin accumulation in each petal stage (B–S) in white-margin-flowered and red-unicolor-flowered carnation cultivars. (A) Petals of each developmental stage in white-margin-flowered ‘Minerva’ and red-unicolor-flowered ‘Berg’. The white bar indicates 2 cm. (B–S) The relative expression of each selected gene at six petal developmental stages. All data represent the average ± SE (three biological replications). DcActin was used as the internal standard. The relative expression level was calculated by setting the value of S6 of ‘Minerva’ as 1. (B) DcbHLH1, (C) DcbHLH2, (D) DcFLS, (E) DcDFR, (F) DcANS, (G) DcF3'H, (H) DcGST, (I) DcMATE, (J) DcAHA10, (K) DcbHLH3, (L) DcMYB1, (M) DcMYB2, (N) DcPAL, (O) DcC4H, (P) Dc4CL, (Q) DcF3H1, (R) DcF3H2, and (S) DcAA5GT-like.
and variegated flowers found in the originally white-flowered '2-241-1 MB' cultivar, and the product of this gene is thought to regulate the expression of LBGs, \( DcDFR \) (Dca4324.1), \( DcANS \) (Dca23371.1), and \( DcGST \) (Dca57804.1) (Totsuka et al., 2018). In the present study, in addition to these three structural genes, the expression levels of four genes involved in flavonoid synthesis or transportation to vacuoles (\( DcFLS \), \( DcF3'H \), \( DcMATE \), and \( DcAHA10 \)) were also shown to be positively correlated with the \( DcbHLH1 \) expression level, especially in red-unicolor-flowered cultivars (Fig. 3). Because red-unicolor-flowered cultivars showed little \( DcbHLH2 \) expression, it is suggested that \( DcbHLH1 \) regulates the transcription of these seven structural genes at least in red-unicolor-flowered cultivars. These structural genes other than \( FLS \) have been reported to be regulated by the MBW complex in several species (Lloyd et al., 2017).

\( DcFLS \) expression levels (Fig. 4) showed no correlation with flavonol accumulation (represented by the peak detected at 350 nm in Fig. 2 and Supplemental Fig. S3) in the central and marginal regions of petals in ‘Minerva’ and ‘Black Jack’. FLS belongs to the class of 2-oxoglutarate-dependent dioxygenases along with \( F3H \) and \( ANS \) (Cheng et al., 2014), and some FLSs exhibit \( F3H \) activity (Lukačin et al., 2003; Prescott et al., 2002;
Sun et al., 2019). Although FLS is reported to be activated by MYB (Li et al., 2020; Mehrtens et al., 2005; Stracke et al., 2007, 2010), and not by bHLH or MBW complexes, F3H is activated by bHLH, which may be a component of an MBW complex (Albert et al., 2020; Jackson et al., 1992; Martin et al., 1991; Morita et al., 2006; Ohno et al., 2011a; Park et al., 2004, 2007). Therefore, DcFLS are postulated to act as an F3H, rather than an FLS, and may be involved in anthocyanin synthesis.

DeMATE and DcAHA10 are orthologs of A. thaliana TT12 and TT13 (AHA10), respectively. TT12 is necessary for the sequestration of the proanthocyanidin (PA) precursor in the vacuole (Debeaujon et al., 2001). TT12 can also mediate the transportation of anthocyanins in vitro (Marinova et al., 2007). TT13 (AHA10) encodes a

![Fig. 4. The relative expression of candidate regulatory genes for anthocyanin accumulation at the petal margin and center at petal stages S4 (A–I) and S6 (A’–I’) in white-margin-flowered and red-unicolor-flowered carnation cultivars. All data represent the average ± SE (three biological replications). DcActin was used as the internal standard. The relative expression level was calculated by setting the value of S4_center of ‘Minerva’ as 1. (A)(A’) DcbHLH1, (B)(B’) DcbHLH2, (C)(C’) DcFLS, (D)(D’) DcDFR, (E)(E’) DcANS, (F)(F’) DcF3’H, (G)(G’) DcGST, (H)(H’) DeMATE, (I)(I’) DcAHA10, (J)(J’) DcAA5GT-like, (K)(K’) DcMYB1 and (L)(L’) DcF3H1.](image-url)
P-type H⁺-ATPase involved in PA biosynthesis and helps the TT12-mediated transportation of PA precursors to vacuoles in the endothelium of the seed coat (Appelhagen et al., 2015; Baxter et al., 2005). PH5 of petunia is an ortholog of AHA10 and is also involved in vacuolar PA accumulation in the seed coat (Verweij et al., 2008). Although both MATE and AHA10 are related to PA, peaks indicating PAs were not detected in any of the cultivars examined by HPLC analysis in the present study (data not shown). Moreover, as shown by RNA-seq in ‘Minerva’, the expression of genes with high homology to BANYULS (BAN) from A. thaliana, which encodes anthocyanidin reductase, an enzyme that converts anthocyanidins to PA (Xie et al., 2003), was almost the same in the central and marginal regions of petals. BAN and PA synthesis are regulated by an MBW complex in Arabidopsis (Baudry et al., 2004), but are not regulated by DcbHLH1 in carnation. DcMATE and DcAHA10 may be involved in the accumulation of anthocyanins rather than PA in carnation petals.

Although the phenylpropanoid pathway is important for the generation of an enormous array of secondary metabolites, including anthocyanins (Vogt, 2010), the expression of DcPAL, DcC4H, and Dc4CL showed no correlation with increased anthocyanin accumulation during petal development (Fig. 3). This suggests that these genes may contribute minimally to anthocyanin synthesis. Ozeki et al. (2020) suggested that another gene encoding PAL, C4H, and 4CL played an important role in anthocyanin synthesis, which was consistent with our results.

Therefore, in carnation, the accumulation of anthocyanins is probably induced by the expression of DcbHLH1 and the structural genes regulated by it, namely, three previously reported genes, DcDFR, DcANS, DcGST, and four newly indicated genes in this paper, DcFLS (which may function as an F3H), DcF3’H, DcMATE, and DcAHA10, at least in red-unicolor-flowered cultivars (Fig. 6).

Repression of two bHLH genes is a key factor in the formation of white petal margins

The expression of DcbHLH1 and the seven structural genes was also positively correlated with anthocyanin accumulation in the central and marginal regions of white-margined flowers at S4 (Fig. 4), suggesting that the repression of DcbHLH1 was involved in the formation of the white margins. DcAA5GT-like also showed a similar expression pattern (Fig. 4), but no positive cor-
relation with the expression of \textit{DcbHLH1} in six petal developmental stages (Fig. 3). This suggests that this gene may not be regulated by \textit{DcbHLH1} and the expression in the central regions of white-margin-flowered cultivars could be increased in another manner. Another bHLH gene, \textit{DcbHLH2}, showed a positively correlated expression pattern with anthocyanin accumulation like \textit{DcbHLH1} only in white-margin-flowered cultivars (Figs. 3 and 4). Moreover, in white-margin-flowered cultivars, the expression pattern of some structural genes, \textit{DcFLS}, \textit{DcF3'H}, \textit{DcMATE}, and \textit{DcAA10}, was more similar to that of \textit{DcbHLH2} than \textit{DcbHLH1} because the expression did not increase rapidly, but rather decreased (Figs. 3 and 4). This decreased expression pattern of \textit{DcbHLH2} and structural genes was especially apparent in the central petal regions of white-margin-flowered cultivars (Fig. 4). On the other hand, the expression of \textit{DcbHLH1} in the same regions increased from S4 to S6 (Fig. 4). The earlier initiation of anthocyanin synthesis (Fig. 3A) may result in its earlier stop in white-margin-flowered cultivars, followed by the decreasing expression of these genes with no correlation between the expression and the anthocyanin accumulation at S6 in the central and marginal regions (Fig. 4). Therefore, \textit{DcbHLH2} rather than \textit{DcbHLH1}, may regulate these structural genes and the anthocyanin accumulation in white-margin-flowered cultivars. Totsuka et al. (2018) reported that the expression of a bHLH gene (Dca38761.1) with high homology to \textit{DcbHLH2} was very low in the petals of the red-flowered carnation variety ‘4-94-1 MR’. Similarly, the expression of \textit{DcbHLH2} was found to be minimal in three red-unicolor-flowered cultivars, ‘Anney’, ‘Berg’, and ‘Spinel’ (Fig. 3). The sequence of \textit{DcbHLH2} identified in this study was longer in the 5’ direction than that of Dca38761.1 and contained a bHLH-MYC\textsubscript{N} domain (PF14215.5), which was not included in the registered ORF sequence of Dca38761.1. We tried to amplify the full-length genomic \textit{DcbHLH2} sequence by PCR and detected bands of the same length in all cultivars (data not shown). This result suggested that some repression of \textit{DcbHLH2} transcription could occur in red-unicolor-flowered cultivars and may be the default situation in carnation petals. bHLH components of MBW complexes, TT8/GL3/EGL3, redundantly regulate the biosynthesis of anthocyanins in \textit{Arabidopsis} (Zhang et al., 2003). In petunia, both \textit{AN1} and \textit{JAF13} regulate anthocyanin biosynthesis by activating structural genes (Koes et al., 2005; Mol et al., 1998; Quattrocchio et al., 1998; Spelt et al., 2000). In \textit{Matthiola incana}, an MBW complex containing MibHLH2 (classified in the same clade as \textit{DcbHLH1}) activates the transcription of anthocyanin biosynthetic genes, and MibHLH1 (belonging to the same clade as \textit{DcbHLH2}) may function as an enhancer of anthocyanin biosynthesis when it coexists with the MBW complex (Nuraini et al., 2020). In other cases, it has been demonstrated that the mutation of a \textit{DcbHLH2} ortholog results in a complete or severe loss of anthocyanin pigmentation, despite the continued expression of the orthologs of \textit{MibHLH1} in petunia (Albert et al., 2011; Spelt et al., 2000), \textit{Ipomoea} (Park et al., 2004), and in dahlia (Ohno et al., 2011a). These results indicate that the orthologs of \textit{DcbHLH2} are the main regulatory genes of anthocyanin biosynthesis in these species. In carnation, \textit{DcbHLH2} is also likely to be involved in anthocyanin synthesis. Therefore, we can conclude that the simultaneous repression of two bHLH genes, \textit{DcbHLH1} and \textit{DcbHLH2}, results in white coloration in the marginal regions of carnation petals by inducing the absence of anthocyanin synthesis.

One of the potential mechanisms of the complete repression of gene expression is PTGS. In petunia and dahlia, flower color patterns consisting of red and white regions result from the PTGS of CHSs in white regions (Koseki et al., 2005; Morita et al., 2012; Ohno et al.,...
The white abaxial surface of petals may be formed by similar regulatory mechanisms to white margins

Generally, anthocyanins accumulate primarily or exclusively in the epidermal cells of flowers (Martin and Gerats, 1993). In red-unicolored carnation cultivars, pigmentation by anthocyanins was observed in both adaxial and abaxial epidermal cells (Fig. 2), which is consistent with previous reports in carnation (Nakayama et al., 2012; Okamura et al., 2013) and many other floricultural species (Deguchi et al., 2020; Jackson et al., 1992; Yoshida et al., 2005). Both adaxial and abaxial epidermal cells are derived from the L1 layer of the shoot apical meristem and are assumed to have common features. However, several phenotypes differ between adaxial and abaxial surfaces; for example, adaxial petals surface generally consist of conical cells, while abaxial surfaces consist of flat cells in many plant species (Christensen and Hansen, 1998; Kay et al., 1981), indicating that different regulation mechanisms operate on each surface. In this study, red pigmentation conferred by anthocyanins was not observed in the petal abaxial epidermal cells of white-margin-flowered cultivars (Fig. 2). The expression of two bHLH genes, DcbHLH1 and DcbHLH2, and seven structural genes, DcFLS, DcDFR, DcANS, DcF3′H, DcGST, DcMATE, and DcAHA10, which was repressed in white marginal regions, was lower in the abaxial epidermal layer than in the adaxial epidermal layer in ‘Minerva’ (Fig. 5). Moreover, the expression levels of DcMYB1 and DcF3′H were higher in the abaxial layer than in the adaxial layer (Fig. 5), which was consistent with the negative correlation between their expression and anthocyanin accumulation found in the comparison of white margins and dark-red centers (Fig. 4). Therefore, the white abaxial surface resulted from the absence of anthocyanin accumulation in the abaxial epidermal layer, and the white petal margins could be regulated by similar mechanisms.

The ‘Nobbio series’ carnation cultivars originated from hybridization between carnation and some allied wild species, including D. chinensis. Some varieties in this series have petal margins with paler reddish coloration (‘Nobbio Burgundy’), pink or light coloration (‘Nobbio Bilzt’), and purple coloration (‘Nobbio Violet’). These phenotypes are classified into a group of marginal picotee patterns with clearer borders (Nakayama, 2020), including ‘Minerva’. All of these ‘Nobbio series’ cultivars commonly have less intensely colored abaxial surfaces and marginal regions. The paler coloration at the margins and on the abaxial surface may be regulated by DcbHLH1 and DcbHLH2 through quantitative repression, rather than the complete repression mechanism shown in the present study, which induces lower accumulation of anthocyanins. Considering that ‘Black Jack’ is also an interspecific hybrid among several species of Dianthus, DcbHLH2 transcription that could be involved in petal margins...
and abaxial surfaces with different colors may originate from related species such as *D. chinensis* L. because it has many variants with white-margined flowers. Moreover, we will have to investigate whether the two phenotypes of white margins and white abaxial surfaces always occur at the same time or whether they can be separated by conducting phenotypic studies of, for example, postmatting generations. Interestingly, no expression of any of the genes examined, including *DeMYB1* and *DeF3H1*, was detected in the inner cell layer of petals (Fig. 5). This result suggested an alternative mechanism inhibiting anthocyanin accumulation in the inner layer of petals that may lead to exclusive accumulation in epidermal cells. In the present study, we analyzed only candidate genes regulating white margins based on RNA-seq results and quantified their expression among cell layers only in ‘Minerava’. To reveal the mechanisms underlying the formation of white abaxial surfaces and the layer-specific accumulation of anthocyanins in petals, further research targeting a wider range of genes and including more cultivars should be conducted. In addition, further experiments, such as in situ hybridization experiments, are necessary to confirm the localized expression of those genes. Because pigmentation is visible and has intrinsic advantages, these experiments may lead to new insights into gene expression systems regarding tissue- and/or cellular-specificity.

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