Analysis of Flower Color Variation in Carnation (Dianthus caryophyllus L.) Cultivars Derived from Continuous Bud Mutations

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Bud-mutation carnation cultivars of the “MINAMI series” have a diversity of flower color in which the directions of bud sports are recorded. ‘Poly Minami’, which is the origin of the “MINAMI series”, produced the eight cultivars with various petal colors through continuous bud mutations. Flavonoid pigments analysis showed that the flower color variation is produced by the difference in the quantitative ratios of pelargonidin-typed anthocyanin and chalcononaringenin 2′-O-glucoside (Ch2′G). Acyanic cultivars; ‘Poly Minami’, ‘Lemon Minami’ and ‘Vanilla Minami’ had Ch2′G showing a yellow coloration as a major flavonoid with different concentrations in the petals. Cyanic cultivars with pinkish petals; ‘Orange Minami’, ‘Minami’, ‘Passion Minami’ and ‘Feminine Minami’ had different ratios of 3,5-di-O-(β-glucopyranosyl) pelargonidin 6′′-O-4,6′′′-O-1-cyclic malate (Pg3,5cMdG), showing a pink coloration, and Ch2′G as major flavonoids in the petals. The variegated cultivar ‘Sakura Minami’, with deep pink sectors and flecks on pale pink petals, accumulated a small amount of Pg3,5cMdG. The red-flowered cultivar ‘Tommy Minami’ accumulated pelargonidin 3-O-malylglucoside (Pg3MG) showing a pink coloration, and Ch2′G as major flavonoids in the petals. The gene expression analysis through flower-bud development showed that the ratios of Pg3,5cMdG and Ch2′G are produced by the difference in the expression levels of flavonoid biosynthesis-related genes; the dihydroflavonol 4-reductase gene (DFR), the chalcononaringenin 2′-O-glucosyltransferase gene (CHGT2) and the chalcone isomerase gene (CHI2) and the acyl-glucose-dependent anthocyanin 5-O-glucosyltransferase gene (AA5GT) and an anthocyanin transportation-related gene; the glutathione S-transferase-like gene (GSTF2). This study revealed that the flower color variations in the “MINAMI series” are caused by genetic and metabolic changes associated with flavonoid biosynthesis and identified five candidate genes for flower color changes in the “MINAMI series”.

Key Words: bud sports, cyanic color, acyanic color, flavonoids, flavonoid biosynthesis-related genes.

Introduction

The carnation (Dianthus caryophyllus L.) is one of the most important ornamental plants in the global flower market. Carnation cultivars have flower color diversity which is an attractive trait for consumers. Carnation cultivars, with various petal colors, have been bred by conventional cross breeding and bud mutation (Holley and Baker, 1991). Although bud mutation is important for the production of novel cultivars, the mechanism of the flower color variation caused by bud mutation remains unknown.

The diversity of flower color in ornamental plants is produced by four major pigments; chlorophyll, carotenoid, betalain and flavonoid (Tanaka et al., 2008). The chlorophyll content of the petals decreases as the flower bud develops (Mayak et al., 1998). However, carnation cultivars with greenish petals can maintain a higher concentration of chlorophyll in the petals under chlorophyll degradation by flower-bud development than non-greenish-flowered carnations because of the higher rate of the chlorophyll biosynthesis through petal maturation (Ohmiya et al., 2014). Carotenoid contributes to the flower coloration in the yellow to red range in many flowering plants, whereas the order Caryophyllales accumulates very low levels of carotenoid in the petals (Ohmiya, 2013). This was also confirmed in Dianthus plants belonging to Caryophyllaceae in the order Caryophyllales (Gatt et al., 1998; Ohmiya et al., 2013).
The flower coloration in the order Caryophyllales is caused by the biosynthesis of betalain; betacyanin and betaxanthine giving red and yellow, respectively (Brockington et al., 2011). However, Molluginaceae and Caryophyllaceae, in the order Caryophyllales, produce flavonoids in the petals instead of betalain (Brockington et al., 2011; Kay et al., 1981).

Flower color variation is produced by the combination of anthocyanins and chalcononaringenin 2′-O-glucoside (Ch2′G) in carnations (Maekawa and Nakamura, 1977) (Fig. 1). “Cyanic color” is caused by the accumulation of four major anthocyanins; pelargonidin 3-O-malylglucoside (Pg3MG), 3,5-di-O-(β-glucopyranosyl) pelargonidin 6‴″-O-4,6‴″-1-cyclic malate (Pg3,5cMdG), cyanidin 3-O-malylglucoside (Cy3MG) and 3,5-di-O-(β-glucopyranosyl) cyanidin 6‴″-O-4,6‴″-1-cyclic malate (Cy3,5cMdG), generally giving red, pink, darkish red or purple, respectively (Bloor, 1998; Nakayama et al., 2000; Terahara and Yamaguchi, 1986). These anthocyanins are synthesized by the anthocyanin malyltransferase (AMalT) and/or the acyl-glucose-dependent anthocyanin 5-O-glucosyltransferase (AA5GT) after transportation of pelargonidin 3-O-gulucoside (Pg3G) and/or cyanidin 3-O-glucoside (Cy3G) into the vacuoles by the glutathione S-transferase-like (GSTM2), resulting in genotype-specific flower coloration (Abe et al., 2008; Matsuba et al., 2012). “Acyanic color” is produced by the accumulations of flavonol/flavone and Ch2′G which are responsible for white, cream and yellow petal colors (Mato et al., 2000; Onozaki et al., 1999; Yoshida et al., 2004). When anthocyanin coexists with Ch2′G in the same cells, orange and bronze petal colors are produced (Geissman and Mehlquist, 1947; Gonnet and Hieu, 1992). The petal color phenotypes of carnations are genetically regulated by flavonoid biosynthesis-related genes and an anthocyanin transportation-related gene (Geissman and Mehlquist, 1947; Mehlquist, 1957; Sasaki et al., 2013) (Fig. S1). To date, it has been reported that the chalcone isomerase gene (CHI), the flavanone 3-hydroxylase gene (F3H), the dihydroflavonol 4-reductase gene (DFR), the flavonoid 3′-hydroxylase gene (F3′H), the anthocyanidin synthase gene (ANS), GSTF2, AMalT and AA5GT are mainly involved in flower color variations in carnations (Dedio et al., 1995; Itoh et al., 2002; Mato et al., 2000, 2001; Momose et al., 2013a, b; Nishizaki et al., 2011). Although the seven genes coding chalcononaringenin 2′-O-glucosyltransferase (CHGT) for the synthesis of Ch2′G from chalcononaringenin were isolated by Ogata et al. (2011) and Togami et al. (2011), the effect on flower color variation has not been reported to date.

The flower color changes of carnations caused by bud mutations have been studied extensively (Imai, 1936; Mato et al., 2000; Momose et al., 2013a, b; Morimoto et al., 2018; Wasscher, 1956). Bud mutations have been reported to be caused by the mobility of transposable elements, which can suppress or activate the expression of flavonoid biosynthesis-related genes, resulting in flower color changes (Itoh et al., 2002; Nishizaki et al., 2011; Momose et al., 2013a, b). A previous study of bud mutations showed that irradiation treatment kills the epidermal cells of the periclinal chimera. Hence, a new epidermis is regenerated from inner core cells (Richter and Singleton, 1955; Sagawa and Mehlquist, 1957). It was determined by a histological analysis that X-ray irradiation induces the necrosis of the outer cells of the shoot apex in a white-flowered carnation ‘White Sim’, a periclinal chimera, and then the tunica is reconstructed, resulting in the production of the carnations with the red and white petal colors from ‘White Sim’ (Sagawa and Mehlquist, 1957).

The aim of this study was to reveal how the flower color variations occurred in bud-mutation carnation cultivars of the “MINAMI series”, which has a wide range of petal colors. First, we determined the major flavonoid pigments in the petals of each cultivar of the “MINAMI series”. Second, we investigated the flavonoid amounts and the expression levels of the major flavonoid biosynthesis-related genes isolated from cultivars of the “MINAMI series” during flower-bud development. Based on these results, we identified candidate genes which thought to be involved in the cause of each bud mutation in the “MINAMI series”.

Materials and Methods

Plant materials

Nine “MINAMI series” cultivars; ‘Poly Minami’
Determination of the amount and the types of flavonoid pigments in petals by HPLC

The dried petals were immersed in 50% acetic acid (v/v) and incubated at room temperature (25°C) for 24 hours in the dark. The extract was passed through a 0.45 μm membrane filter (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). The filtered extract was injected into an HPLC system. The HPLC system was constructed with a CBM-20Alite system controller (Shimadzu Corp., Kyoto, Japan), an SPD-M10Avp detector (Shimadzu Corp.), an SIL-10AF auto injector (Shimadzu Corp.), a DGU-12A degasser (Shimadzu Corp.), two LC-20AT pumps (Shimadzu Corp.) and two inertsil ODS-3 columns (3.0 mm × 50 mm and 3.0 mm × 250 mm, GL Sciences Inc., Tokyo, Japan). The column temperature was maintained at 40°C by a CTO-20A column oven (Shimadzu Corp.). Linear gradient elution for 40 min from 25 to 85% solvent B (0.1% trifluoroacetic acid, 20% acetic acid and 25% acetonitrile in H$_2$O, v/v) in solvent A (0.1% trifluoroacetic acid in H$_2$O, v/v) was used as the solvent system. A flow rate of 0.4 mL·min$^{-1}$ was maintained. Anthocyanins and Ch2G were detected based on the absorption at 510 nm and 360 nm, respectively. The type of flavonoids in the petals was identified by comparing their retention times with authentic standards; Cy3MG, Cy3,5cMdG, Pg3MG and Pg3,5eMdG which were extracted from carnations bearing dark red, deep purple, red and deep pink flowers, respectively (Bloor, 1998; Nakayama et al., 2000; Terahara and Yamaguchi, 1986) and Ch2G extracted from the petals of a yellow-flowered cyclamen (Miyajima et al., 1991). The chalcones and flavonols without Ch2G (ChFl; almost all of the contents were flavonols) were calculated by subtracting the peak area of Ch2G from the total peak area on the basis of the 360 nm absorption.

Cloning of flavonoid biosynthesis-related genes

To prepare for the gene expression analysis, flavonoid biosynthesis-related genes were isolated as follows; the phenylalanine ammonia-lyase gene (PAL) and the chalcone synthase gene (CHS) from ‘Orange Minami’, CHI1 and CHI2 from ‘Orange Minami’ and ‘Feminine Minami’, CHGT1 from ‘Orange Minami’, CHGT2 from ‘Feminine Minami’, F3H, DFR, ANS, AmALT and AAGST from ‘Orange Minami’ and an anthocyanin transportation-related gene, GSTF2, was isolated from ‘Feminine Minami’. To confirm the gene sequences, CHI1, CHI2 and DFR were also isolated from ‘Poly Minami’. Ubiquitin 5 gene (UBQ5) was isolated to use as an internal control of quantitative real-time PCR (qRT-PCR) from ‘Orange Minami’ in reference to the research paper of Nomura et al. (2012). Gene-specific primers were designed on the basis of the known sequence of NCBI (https://www.ncbi.nlm.nih.gov/) and the genome sequence of Carnation DB (http://carnation.kazusa.or.jp/) (Table S1). The total RNA was prepared from the frozen petals at stage 2 or 5 according with the modified cetyl trimethyl ammonium bromide (CTAB) method (Chang et al., 1993). cDNA was synthesized from the total RNA by using PrimeScript reverse transcriptase (Takara Bio Inc., Shiga, Japan) and the GeneRacer Oligo dT primer: 5’-GCTGTCAAA

Five stages of flower-bud development

1. closed buds; 2. bud break; 3. top of inflorescence opened; 4. vertically elongated flowers; 5. fully opened flowers. “Pippi Minami” was not analyzed in this research.
Expression analysis by qRT-PCR

Total RNA was extracted from the frozen petals at each flower-bud developmental stage according to the modified CTAB method. To remove contamination by genomic DNA, the total RNA was treated with DNase I of the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized from the total RNA by the PrimeScript RT reagent Kit (Perfect Real Time) (Takara Bio Inc.).

Gene expression analysis by qRT-PCR was performed by using SYBR Premix Ex Taq II (Takara Bio Inc.) and a TaKaRa Thermal Cycler Dice Real-Time System TP-800 (Takara Bio Inc.) according to the manufacturer’s instructions. The specific primer sets for qRT-PCR are listed in Table S2. The PCR reaction was performed under the following conditions: at 95°C for 30 sec, 40 cycles (at 95°C for 5 sec, at 60°C for 30 sec), 1 cycle (at 95°C for 15 sec, at 60°C for 30 sec, at 95°C for 15 sec). PCR programs of CHI2 and DFR are listed in Table S3. UBQ5 was used as an internal control. The primer set for the detection of both DFR1 (acc. no. LC377195) and DFR2 (acc. no. LC377270) was designed.

Results

Flavonoid pigments in the “MINAMI series”

To determine the major flavonoid pigments in the petals, HPLC analysis was carried out by using fully opened flowers. In the five cyanic cultivars, except ‘Tommy Minami’, Pg3,5cMdG and an undetermined anthocyanin (An1) were detected as the major peak and the secondary peak, respectively (Fig. 3). The five cyanic cultivars also had a very small amount of Pg3MG in the petals (Figs. 4A and S2). The three cyanic cultivars, ‘Orange Minami’, ‘Passion Minami’ and ‘Feminine Minami’, had a very small amount of Cy3,5cMdG (Fig. S2). However, Cy3MG was not detected in the five cyanic cultivars (Figs. 3 and S2). The major peak and the secondary peak of ‘Tommy Minami’ were Pg3MG and Cy3MG, respectively (Fig. 3). ‘Tommy Minami’ also had very small amounts of Pg3,5cMdG and Cy3,5cMdG (Fig. S2). On the other hand, no anthocyanins were detected in ‘Poly Minami’ and ‘Lemon Minami’, whereas Pg3,5cMdG was detected in ‘Vanilla Minami’ (Fig. 4A). Ch2′G accumulated in the petals of all cultivars (Fig. 4A).

‘Poly Minami’, with pale yellow petals (Fig. 1), accumulated Ch2′G in the petals as a major flavonoid pigment (Fig. 4A). Three cultivars; ‘Lemon Minami’, ‘Vanilla Minami’ and ‘Orange Minami’ were derived from ‘Poly Minami’ by bud mutations (Fig. 1), ‘Lemon Minami’, with more yellowish petals, accumulated a larger amount of Ch2′G, and the amount of ChFl was markedly smaller than ‘Poly Minami’ (Fig. 5). An off-white-flowered cultivar ‘Vanilla Minami’ accumulated approximately one-tenth the amount of Ch2′G as compared with ‘Poly Minami’. The amount of ChFl in ‘Vanilla Minami’ was significantly larger than ‘Poly Minami’. ‘Vanilla Minami’ showed accumulation of Pg3,5cMdG unlike ‘Poly Minami’ and ‘Lemon Minami’, although the amount was much less than the five cyanic cultivars. ‘Orange Minami’, with bicolor petals showing pink and yellow colorations (Fig. 1), mainly accumulated Pg3,5cMdG and An1 as the major anthocyanins (Fig. 3). In addition, the amounts of Ch2′G and ChFl accumulated in the petals of ‘Orange Minami’ were similar to those of ‘Poly Minami’.

Three cultivars; ‘Minami’, ‘Passion Minami’ and ‘Feminine Minami’ were derived from ‘Orange
Minami’ by bud mutations (Fig. 1). ‘Minami’, with yellowish orange petals (Fig. 1), accumulated a smaller amount of anthocyanin and a larger amount of Ch2′G than ‘Orange Minami’ (Fig. 4A), and the amount of ChFl was smaller than ‘Orange Minami’ as with ‘Lemon Minami’ (Fig. 5). ‘Feminine Minami’ and ‘Passion Minami’ accumulated a larger amount of anthocyanin and a smaller amount of Ch2′G as compared with ‘Orange Minami’. However, neither of these cultivars showed a significant difference in the ChFl amount in the petals as with ‘Orange Minami’. ‘Passion Minami’ with pinkish orange colored petals accumulated a larger amount of Ch2′G than ‘Feminine Minami’ with petals showing only pink coloration because the petal color phenotype showed bicolor petals displaying pink and yellowish colorations (Fig. 1), whereas the yellowish part was smaller than ‘Orange Minami’ (Fig. 1).

Two cultivars; ‘Sakura Minami’ and ‘Tommy Minami’ were derived from ‘Feminine Minami’ (Fig. 1). ‘Sakura Minami’ showed one-fourth the amount of anthocyanin as compared with ‘Feminine Minami’, whereas the amount of Ch2′G did not differ markedly from that of ‘Feminine Minami’ (Fig. 4A). The amount of ChFl was larger in the petals of ‘Sakura Minami’ than ‘Feminine Minami’ (Fig. 5). The petals of ‘Tommy Minami’ were more reddish than ‘Feminine Minami’ as determined by a color meter (Morimoto et al., 2018). Only ‘Tommy Minami’ in the “MINAMI series” accumulated Pg3MG, showing a red coloration, as the major anthocyanin (Fig. 3). The amount of Pg3MG in ‘Tommy Minami’ was larger than ‘Feminine Minami’, whereas the amount of Ch2′G did not differ markedly from that of ‘Feminine Minami’. The amount of ChFl of ‘Tommy Minami’ was also a larger as compared with that of ‘Feminine Minami’.

Fig. 4. Major flavonoids accumulation in the fully opened flowers (A) and at five developmental stages (B) in nine “MINAMI series” cultivars. The amounts of major flavonoids are shown as a relative value. The peak area of HPLC is divided by the fresh weight (g) of the sample. The amounts of Ch2′G of ‘Poly Minami’ and Pg3,5cMdG of ‘Feminine Minami’ at stage 5 are defined as 100, respectively. The relative amount of Pg3MG in ‘Tommy Minami’ is shown as compared with that of Pg3,5cMdG in ‘Feminine Minami’. n.d.; not-detected. Error bars show standard errors of three biological replications (n = 3).
Change in the amount of flavonoid pigments in the petals during flower-bud development

To investigate changes in the accumulated amount of the major flavonoids during flower-bud development, Pg3,5cMdG or Pg3MG and Ch2′G were compared among cultivars. We used five flower-bud developmental stages (Fig. 2). The petals of stage 1 were not colored in cyanic cultivars since anthocyanin did not accumulate (Fig. 4B), and showed the same petal color in all cultivars (data not shown). After bud break, the flower color intensity in the cyanic cultivars increased due to anthocyanin accumulation according to the flower-bud development (Figs. 2 and 4B). Pg3,5cMdG or Pg3MG was not detected at stage 1 in any cultivars (Fig. 4B). Other anthocyanins were not detected at this stage either (data not shown). However, the accumulation of Ch2′G had already started. The accumulative amount of Pg3,5cMdG or Pg3MG increased rapidly at stages 2 to 3 and stages 4 to 5 in five cyanic cultivars without ‘Minami’. The accumulation of Ch2′G also showed a tendency to increase at stages 2 to 3 and stages 4 to 5 in five cyanic cultivars without ‘Minami’.

Isolation of flavonoid biosynthesis-related genes

The flavonoid biosynthesis-related genes isolated form the “MINAMI series” cultivars indicated a close similarity to those of GenBank and Carnation DB. The DDBJ accession numbers of the isolated gene sequences were shown as follows: PAL, LC377188; CHS, LC377191; CHGT1, LC377271; CHGT2, LC377272; F3H, LC377194; ANS, LC377196; AMalT, LC377198; AASGT, LC377199; GSTF2, LC377197; UBQ5, LC377200. The base sequences of CHI1 and DFR differed from known sequences.

The base sequences of the CHI1 genes isolated from ‘Orange Minami’ (acc. no. LC377192) and ‘Feminine Minami’ (acc. no. LC377265) had very short ORF resulting from the footprint. The footprint was present within the first exon of the isolated CHI1 genes. The CHI1 was also isolated from ‘Poly Minami’ (acc. no. LC377264), and then the base sequences of the CHI1 genes having the footprint in the three cultivars were compared with the known CHI1 genes derived from the other carnation cultivars. The results showed that the base sequences of the CHI1 genes of the three cultivars showed a perfect match and the footprint corresponded to that of the gDicCHI1 derived from ‘7019-0’ reported by Itoh et al. (2002) (Fig. S3). The footprint of the gDicCHI1 is known to cause a frameshift mutation (Itoh et al., 2002). Thus, the CHI1 genes of the three cultivars also showed to stop translation at 29 amino acids lacking the conserved domains and the specific residues (Jez and Noel, 2002; Jez et al., 2000; Shimada et al., 2003) (data not shown).

The base sequences of the CHI2 genes isolated from ‘Orange Minami’ (acc. no. LC377193) and ‘Feminine Minami’ (acc. no. LC377269) did not have footprints. The CHI2 of ‘Poly Minami’ (acc. no. LC377268) did not have a footprint either. The base sequences of the CHI2 genes of these three cultivars showed 98.6% homology, as compared with DcCHI2 (Dca60978 from Carnation DB) and the amino acid sequences of the CHI2 genes had conserved residues, such as the active sites associated with substrate specificity and the hydorgen bond network (Jez and Noel, 2002; Jez et al., 2000; Shimada et al., 2003) (Fig. S4).

The base sequences of the DFR genes isolated from ‘Orange Minami’ and ‘Feminine Minami’ were a perfect match and had the same sequences as the 26 bp existing within the DpDFR of Dianthus plumarius, whereas the known DFR (acc. no. Z67983) did not have the same sequences (Fig. S5). The isolation of DFR from ‘Poly Minami’ was performed with the primer set used for the isolation of DFR from ‘Orange Minami’ and ‘Feminine Minami’ (Table S1). The result showed that the base sequence of the DFR isolated from ‘Poly Minami’ was different from those of ‘Orange Minami’ and ‘Feminine Minami’; it had of a target site duplication-like sequence concerning insertion of a transposon or a footprint-like sequence concerning excision of a transposon (Fig. 6). Therefore, the DFR genes isolated from ‘Orange Minami’ and ‘Feminine Minami’ were named DFR1 (acc. no. LC377195), and the DFR isolated from ‘Poly Minami’ was named DFR2 (acc. no. LC377270). The DFR2 isolated from ‘Poly Minami’ showed high homology with that of the gDicDFR1 isolated from ‘Rhapsody’ (Itoh et al., 2002). The base sequence of the DFR2 showed approximately 98% homology with the DFR1 isolated from ‘Orange Minami’. The predicted protein of the isolated DFR2, which encodes 29 amino acids by a frameshift mutation, lacked the conserved domains and the regions of
Expression pattern of flavonoid biosynthesis-related genes during flower-bud development

The expression level of \textit{PALLI} tended to increase slightly through the development of flower buds (Fig. 7). The expression level of \textit{CHS} tended to be high at stages 1 and 2 and decreased at stages 3 and 4. Furthermore, at stage 5, the expression level of \textit{CHS} was high in all cultivars. \textit{CHI2} and \textit{F3H} were also expressed at stages 1 and 2, and then those expression levels decreased from stages 3 to 5. The expression levels of \textit{DFR}, \textit{ANS}, \textit{GSTF2}, \textit{AMaT} and \textit{AA5GT} were low at stage 1 in all cultivars, and then these genes were expressed highly at stages 2 to 5, unlike \textit{CHI2} and \textit{F3H}. The expression pattern of \textit{AA5GT} was a little different from those of \textit{DFR} and \textit{ANS}. The expression level of \textit{AA5GT} was low at stage 3 and increased at stage 4 in all cultivars.

\textit{CHGT1} was expressed highly at stage 1 in cultivars accumulating a large amount of anthocyanin and at stage 5 in seven cultivars except ‘Minami’ and ‘Tommy Minami’, whereas the expression level was very low at stages 2 to 4 in all cultivars (Fig. 7). The expression pattern of \textit{CHGT2} differed from that of \textit{CHGT1}. The expression pattern of \textit{CHGT2} was similar to those of late biosynthetic genes such as \textit{DFR} and \textit{GSTF2}. In

substrate specificity (Beld et al., 1989; Johnson et al., 2001) (data not shown).
addition, CHGT2 showed a tendency toward a low expression level through flower-bud development in cyanic cultivars except 'Orange Minami'.

**Comparison of gene expression levels among nine “MINAMI series” cultivars**

We compared the expression levels of flavonoid biosynthesis-related genes among bud-mutation cultivars (Fig. 7). ‘Poly Minami’, as the origin of the “MINAMI series” (Fig. 1), showed very low expression of DFR at all stages of flower-bud development. ‘Lemon Minami’ and ‘Vanilla Minami’ derived from ‘Poly Minami’ also showed a very low expression level of DFR through flower-bud development. In ‘Lemon Minami’, the expression level of CHI2 was significantly lower at stages 1 and 2 than ‘Poly Minami’. In ‘Vanilla Minami’, the expression levels of PAL, CHS, CHI2, F3H, AMaT, AA3GT and GSTF2 tended to be higher at stages 1 and 2 as compared with ‘Poly Minami’. ‘Orange Minami’ showed a higher expression level of DFR than ‘Poly Minami’. In addition, the expression level of CHI2 at stage 2 was lower than ‘Poly Minami’.

In ‘Minami’ derived from ‘Orange Minami’ (Fig. 1), CHI2 showed a lower expression level than that of ‘Orange Minami’ at stages 1 and 2 the same as ‘Lemon Minami’ (Fig. 7). In ‘Passion Minami’ derived from ‘Orange Minami’, CHI2 was expressed more highly at stage 1 as compared with ‘Orange Minami’. The expression level of CHGT2 was markedly lower at all developmental stages. In ‘Feminine Minami’ derived from ‘Orange Minami’, CHI2 was expressed more highly at stages 1 and 2 than that of ‘Orange Minami’. The expression level of CHGT2 of ‘Feminine Minami’ was lower at stages 3 to 5 than that of ‘Orange Minami’, whereas it was higher than ‘Passion Minami’ at all developmental stages.

In ‘Sakura Minami’ derived from ‘Feminine Minami’ (Fig. 1), the expression level of GSTF2, an anthocyanin transportation-related gene, tended to be lower, particularly at stage 3 (Fig. 7). ‘Sakura Minami’ has deep pink sectors and flecks on pale pink petals (Fig. 1). Therefore, we investigated the amounts of total anthocyanin and ChFl and the expression level of GSTF2 in the deep pink sectors and the pale pink regions of fully opened flowers. The deep pink sectors of ‘Sakura Minami’ showed a markedly larger amount of total anthocyanin as compared with pale pink regions (Fig. 8A), whereas the amount of ChFl showed no difference between the deep pink sectors and the pale pink regions of ‘Sakura Minami’ (Fig. 8B). In addition, the expression level of GSTF2 was significantly higher in the deep pink sectors of ‘Sakura Minami’ than the pale pink regions and similar to that of ‘Feminine Minami’ (Fig. 8C). These results indicate that the flower color of ‘Sakura Minami’ is linked to the reduction of GSTF2 expression in the pale pink regions. ‘Tommy Minami’, derived from ‘Feminine Minami’, expressed AA3GT in the petals. It showed a lower expression level through flower-bud development, particularly at stages 1 and 2, than ‘Feminine Minami’.

**Discussion**

**Relationship between flavonoid accumulation and gene expression through flower-bud development**

The bud-mutation carnation cultivars in the “MINAMI series” consist of various flower color genotypes which originated from one genotype, ‘Poly Minami’, and this derivation history has been clearly documented (Morimoto et al., 2018). The present study revealed that the petal color variation of the “MINAMI series” is determined by the composition and the quantity of accumulative flavonoids in the petals (Fig. 4A). Moreover, we revealed the candidate genes responsible for flower color mutation. No cultivars in the “MINAMI series” accumulated anthocyanins in the petals of closed buds, showing no coloration due to very low expression levels of late biosynthetic genes such as DFR and GSTF2 (Figs. 2, 4B and 7). On the other hand, all cultivars had already started accumulating Ch2′G before flower buds break due to the combination of the expression of early biosynthetic genes such as PAL and CHS and the glycosylation of chalconoraringenin by CHGT activity (Fig. 7). The expression of CHGT1/2 at stage 1 may be related to the accumulation of Ch2′G, whereas there was no clear correlation between the amounts of Ch2′G and the expression levels of CHGT1/2 at stages 2 to 5.
The expression of CHGT1/2 in closed buds could affect the accumulation of Ch2′G from bud break. It has been reported that carnation cultivars have seven CHGTs (Ogata et al., 2004; Togami et al., 2011). Furthermore, carnations have over ten types of CHGTs according to Carnation DB. Therefore, the other CHGTs, except the isolated CHGT1/2 in this research, are likely related to the accumulation of Ch2′G in the “MINAMI series”. The increases in anthocyanin and Ch2′G contents in the petals were caused by the expression of the late biosynthetic genes and CHGTs, respectively. We found that the cultivar-specific-composition ratio of anthocyanin and Ch2′G did not change during flower-bud development (Fig. 4B). This indicated that the composition ratio of anthocyanin and Ch2′G, identifying the specific phenotype, is determined genetically at the early stage of flower-bud development.

Genetic derivation of the “MINAMI series”

‘Poly Minami’, the origin of the “MINAMI series” cultivars (Fig. 1), accumulated Ch2′G in the petals as the major flavonoid pigment without anthocyanin (Fig. 4A), resulting in a pale yellow coloration (Fig. 1). As with the accumulation of Ch2′G, the inactivation of DFR is also needed for the yellow coloration in the carnation petals because chalcononaringenin is non-enzymatically converted to naringenin, a precursor for anthocyanin biosynthesis (Itoh et al., 2002; Moustafa and Wong, 1967; Spribille and Forkmann, 1982; Stitch et al., 1992). In fact, the expression of DFR was suppressed at all stages of flower-bud development in yellow carnation cultivars in the “MINAMI series” (Fig. 7). It has been reported that the sequence mutation of CHI or the repression of CHI transcripts may decrease the conversion of chalcononaringenin to naringenin and promote the synthesis of Ch2′G from chalcononaringenin (Itoh et al., 2002; Yoshida et al., 2004). Thus, we isolated two CHI genes from the petals of three cultivars in the “MINAMI series” with the reference of Carnation DB. In Carnation DB, CHI1 (Dca60979) localized close to CHI2 (Dca60978) on the same scaffold 94 (Miyahara et al., 2018). ‘Poly Minami’ had two types of CHI, CHI1 coding an inactive enzyme caused by the footprint as mentioned above and CHI2 coding an active enzyme (Figs. S3 and S4). This fact suggests that CHI2 converts chalcononaringenin to naringenin because flavonols accumulate in the petals of ‘Poly Minami’ (Fig. 5). Therefore, these results demonstrated that the blocking of anthocyanin biosynthesis by the suppression of DFR expression is one of the causes of ‘Poly Minami’ with pale yellow petals.

Lemon Minami’, derived from ‘Poly Minami’ (Fig. 1), has more yellowish petals than ‘Poly Minami’ due to a larger amount of Ch2′G in the petals (Figs. 1 and 4A). It is assumed that ‘Lemon Minami’ accumulated the larger amount of Ch2′G because the synthesis of Ch2′G from chalcononaringenin by CHGTs was given priority over the conversion to naringenin due to the low expression level of CHI2 (Fig. 7). Therefore, the amount of ChFl was smaller in the petals of ‘Lemon Minami’ than ‘Poly Minami’ (Fig. 5). Although the Ch2′G content is related to the intensity of the yellow petal color, the factors that regulate the concentration of Ch2′G have not been identified (Yoshida et al., 2004). Our results suggest that the suppression of CHI2 transcription is one of the causes of the change in intensity of the yellow petal color.

‘Vanilla Minami’, derived from ‘Poly Minami’, accumulated a much smaller amount of Ch2′G as compared with ‘Poly Minami’ (Fig. 4A), resulting in an off-white coloration in the petals (Fig. 1). However, the results of gene expression analysis could not explain the genetic mechanism of the derivation of ‘Vanilla Minami’ from ‘Poly Minami’, since the expression levels of CHI2 and CHGT1/2, which are involved in the concentration of Ch2′G from chalcononaringenin and accelerate the conversion of chalcononaringenin to naringenin because the amount of ChFl in ‘Vanilla Minami’ was larger than ‘Poly Minami’ (Fig. 5). Currently, expression analysis of the other CHGTs is in progress.

‘Orange Minami’, derived from ‘Poly Minami’ (Fig. 1), produced a similar amount of Ch2′G in the petals as ‘Poly Minami’ (Fig. 4A). In addition, ‘Orange Minami’ accumulated anthocyanin in the petals because DFR1 was expressed more highly than that of ‘Poly Minami’ (Fig. 7). Thus, ‘Orange Minami’ accumulated both anthocyanin and Ch2′G (Fig. 4A), with bicolor petals consisted of pink distal and yellowish orange proximal regions (Fig. 1). First, we assumed that the derivation of ‘Orange Minami’ from ‘Poly Minami’ was caused by restoring the expression of DFR1 due to the excision of the transposable element as mentioned by Itoh et al. (2002). However, there was no footprint in the base sequences of the DFR1 genes isolated from ‘Orange Minami’ and ‘Feminine Minami’. To confirm whether DFR1 of ‘Poly Minami’ has normal base sequences, we carried out isolation of DFR1 from ‘Poly Minami’. The result of the sequencing analysis showed that the DFR of ‘Poly Minami’ (DFR2) had the sequence of a target site duplication-like or a footprint-like, showing high homology with a footprint of the gDicDFR isolated from ‘Rhapsody’ (Itoh, et al., 2002) (Fig. 6). The normal DFR1 sequence could not be isolated from ‘Poly Minami’ by RT-PCR. However, genomic analysis showed that ‘Poly Minami’ had
genomic DFR1 and DFR2 in the petals (Fig. 9). In the present study, we could not confirm that DFR1 and DFR2 are allelic. These results suggest that genomic DFR1 is absent only in the epidermis of ‘Poly Minami’, causing anthocyanin accumulation, or that the expression of the DFR1 is down-regulated by mutation of active transcription factors or repressors.

‘Minami’, derived from ‘Orange Minami’ (Fig. 1), had smaller anthocyanin and larger Ch2′G amounts in the petals than ‘Orange Minami’ (Fig. 4A). Gene expression analysis indicated that the difference in the composition of Pg3,5cMdG and Ch2′G between the two cultivars was caused by the lower expression level of CH12 in ‘Minami’ as in ‘Lemon Minami’ (Fig. 7). The lower expression level of CH12 decreased the conversion of chalcononaringenin to naringenin, resulting in a reduction in the ChFI amount as in ‘Lemon Minami’ (Fig. 5). Moreover, a low expression level of CH12 promotes the accumulation of Ch2′G, but does not completely block the conversion of chalcononaringenin to naringenin because of the non-enzymatic reaction as in other carnations (Spribille and Forkmann, 1982). Therefore, the single yellowish-orange petals of ‘Minami’ are produced by a small amount of anthocyanin and a large amount of Ch2′G caused by sufficient DFR expression and the suppression of CH12, respectively (Figs. 1, 4A and 7).

In ‘Passion Minami’, derived from ‘Orange Minami’ (Fig. 1), the expression level of CHGT2 was markedly lower than the other cultivars during flower-bud development (Fig. 7). In addition, ‘Passion Minami’ showed that a higher expression level of CH12 at stage 1 as compared with ‘Orange Minami’. Thus, the low activity of CHGT2 and the high activity of CH12 were considered to accelerate the conversion of chalcononaringenin to naringenin, resulting in accumulation of a larger amount of anthocyanin and a smaller amount of Ch2′G (Fig. 4A). Yoshida et al. (2004) investigated the relationship between the S gene regulating anthocyanin amount and the concentration of Ch2′G involved in the intensity of the yellow petal color, however they could not find the regulation factors of the Ch2′G amount. In this study, the variation in the Ch2′G content may have contributed to the intensity of the yellow petal color by regulating CH12 and CHGT2 expressions.

‘Feminine Minami’, derived from ‘Orange Minami’ (Fig. 1), accumulated a larger amount of Pg3,5cMdG and a smaller amount of Ch2′G as compared with ‘Orange Minami’ (Fig. 4A). The derivation could be explained by the high expression level of CH12 at early developmental stages and the low expression level of CHGT2 at late developmental stages as mentioned above in the derivation of ‘Passion Minami’. ‘Feminine Minami’ also expressed inactive CH11 highly, the same as ‘Vanilla Minami’ (Fig. S6), but we could not elucidate why the expression level of CH11 was significantly higher than the derivative parent. For an analogy of the gene expression analysis for CHIs and CHGTs, the regulator for both expression of CHIs and CHGTs may be involved in bud mutations of the “MINAMI series” cultivars.

In ‘Sakura Minami’, derived from ‘Feminine Minami’ (Fig. 1), the expression level of GSTF2 tended to be lower than ‘Feminine Minami’ during flower-bud development (Fig. 7). Moreover, the expression level of GSTF2 was markedly higher in the deep pink sectors, accumulating a large amount of anthocyanin (Fig. 8A), as compared with the pale pink regions (Fig. 8C). The GSTF2 enzyme is responsible for the intensity of the cyanic petal color in carnations by the regulation of anthocyanin transportation into vacuoles (Momose et al., 2013a; Sasaki et al., 2012). In the variegated carnations, the suppression of CHGT2 and the high activity of CH12 were consid-
Fig. 10. Relationship with bud mutations and the expression of flavonoid biosynthesis-related genes in the “MINAMI series”. The derivation genealogy of the “MINAMI series” is combined with the up-regulation (up) and down-regulation (dwn) of the candidate genes for bud mutations.

2. ‘Lemon Minami’
1. ‘Poly Minami’
3. ‘Vanilla Minami’
4. ‘Orange Minami’
5. ‘Minami’
6. ‘Passion Minami’
7. ‘Feminine Minami’
8. ‘Sakura Minami’
9. ‘Tommy Minami’

Table 1. Gene expression analysis of the cultivars in the “MINAMI series”.

| Cultivar    | Expression of Candidate Genes |
|-------------|--------------------------------|
| ‘Poly Minami’ | CHI2 up, CHGT2 dwn, GSTF2 up |
| ‘Vanilla Minami’ | CHI2 dwn, GSTF2 up |
| ‘Orange Minami’ | CHI2 dwn, CHGT2 up |
| ‘Minami’ | GSTF2 dwn, AA5GT up |
| ‘Sakura Minami’ | GSTF2 dwn, AA5GT up |
| ‘Tommy Minami’ | GSTF2 dwn, AA5GT up |

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