Introduction

Carotenoids are important pigments responsible for petal colors ranging from yellow to red. The main role of carotenoids in flowers is the attraction of insect and bird pollinators. In the green tissues of higher plants, carotenoids have important functions in photosynthesis and protection against photooxidative damage (Robert et al., 2004, Ruban et al., 2007). There is a wide variation in carotenoid composition in petals among plant species; most carotenoids are xanthophylls (Kishimoto et al., 2007, Ohmiya 2011). Carotenoids in petals are biosynthesized through multiple enzymatic steps and accumulate in chromoplasts (Cazzonelli and Pogson 2010, Hirschberg 2001). The condensation of two molecules of geranylgeranyl diphosphate in the first committed and rate-limiting step produces phytoene. Through desaturation, isomerization, and cyclization steps, α- and β-carotene are produced via lycopene. These carotenoids from phytoene to α- and β-carotene are “carotenes” defined as carotenoids composed only of hydrogen and carbon atoms. The hydroxylation of α- and β-carotene produces “xanthophylls”, defined as carotenoids containing oxygen atoms, and those are followed by epoxidation. A number of studies showed that carotenoid accumulation in petals is regulated at the transcriptional level of carotenoid biosynthetic genes (reviewed by Ohmiya 2013). Overall carotenoid accumulation is regulated through degradation by carotenoid cleavage dioxygenase (CCD) family enzymes (Ohmiya 2009, Ohmiya et al. 2006).

In green leaves, all xanthophylls occur in free form, while in petals they are mostly esterified (Ariizumi et al., 2014, Breithaupt et al. 2002, Goodwin 1980, Maoka et al., 2011, Yamamizo et al. 2010). In some carotenoid-rich petals and fruits, various types of carotenoid-containing bodies, including fibrillar, globular, and tubular, are formed inside the chromoplasts (Camara et al. 1995, Ljubesić et al. 1991). Deruère et al. (1994) reported that esterified xanthophylls were more efficient for fibril assembly than free xanthophylls in vitro. They presumed that carotenoid sequestration by the formation of carotenoid-containing bodies prevents harmful effects of excess carotenoids on cellular functions. Recently, Ariizumi et al. (2014) identified a gene encoding xanthophyll esterase (XES) in the pale yellow petal 1 (pyp1) mutants of tomato. They showed that disruption of PYP1 caused complete loss of esterified xanthophylls, reduction in

Comparison of petunia and calibrachoa in carotenoid pigmentation of corollas

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Petunia (Petunia hybrida) is an important ornamental plant with a wide range of corolla colors. Although pale-yellow-flowered cultivars, with a low amount of carotenoids in their corollas, are now available, no deep-yellow-flowered cultivars exist. To find why petunia cannot accumulate enough carotenoids to have deep-yellow flowers, we compared carotenoid profiles and expression of carotenoid metabolic genes between pale-yellow-flowered petunia and deep-yellow-flowered calibrachoa (Calibrachoa hybrida), a close relative. The carotenoid contents and the ratios of esterified xanthophylls to total xanthophylls in petunia corollas were significantly lower than those in calibrachoa, despite similar carotenoid components. A lower esterification rate of trans-xanthophylls than of cis-xanthophylls in petunia suggests that petunia xanthophyll esterase (XES) has low substrate specificity for trans-xanthophylls, which are more abundant than cis-xanthophylls in petunia corolla. The expression of genes encoding key enzymes of carotenoid biosynthesis was lower and that of a carotenoid catabolic gene was higher in petunia. XES expression was significantly lower in petunia. The results suggest that low biosynthetic activity, high cleavage activity, and low esterification activity cause low carotenoid accumulation in petunia corollas.

Key Words: Calibrachoa hybrida, carotenoid, flower color, gene expression, Petunia hybrida, xanthophyll esterase.
We show that low expression of carotenoid biosynthetic genes, high expression of a carotenoid catabolic gene, and low esterification activity are the main reasons for the low carotenoid accumulation in petunia corollas.

Materials and Methods

Plant materials

Pale-yellow-flowered petunia ‘California Girl’ and deep-yellow-flowered calibrachoa ‘Tifosi Deep Yellow’ were grown in greenhouses at the NARO Institute of Vegetable and Floriculture Science (Tsukuba, Japan). Tubes and limbs of corollas on flowering day and mature leaves were sampled (Fig. 1A–1C) and stored at –80°C until use.

Cloning of carotenoid metabolic genes

We isolated partial-length cDNAs of carotenoid metabolic genes and actin of calibrachoa, and full-length cDNAs encoding putative xanthophyll esterase (XES), an ortholog of tomato PYP1 (SlPYP1), of both species, as described by Kishimoto et al. (2018). Primer sequences are shown in Supplemental Tables 1 (RT-PCR) and 2 (RACE of XES). cDNA sequences were deposited in DDBJ/EMBL/GenBank under the following accession numbers: 1-deoxy-

Fig. 1. (A) Flowers of petunia ‘California Girl’ and calibrachoa ‘Tifosi Deep Yellow’. (B) Vertical sections of corollas. (C) Sampling positions of tube and limb. (D) Carotenoid content in tubes, limbs, and leaves of petunia and calibrachoa. (E) Ratio of esterified xanthophylls to total xanthophylls. Analyses were performed in triplicate; means ± SE are shown. The same letter indicates no significant difference by Tukey’s test (P < 0.05).
Carotenoids in petunia and calibrachoa

Results

Comparison of carotenoid content and composition between pale-yellow-flowered petunia and deep-yellow-flowered calibrachoa

The total average carotenoid content of calibrachoa was 6.9× that of petunia in tubes, 11.0× in limbs, and 1.7× in leaves (Fig. 1D). Carotenoid components in saponified samples of limbs and tubes were similar between petunia and calibrachoa; we detected mainly (all-β)-neoxanthin, (all-ε)-violaxanthin, (all-ε)-lutein, (all-ε)-zeaxanthin, and (all-ɛ)-β-carotene (Fig. 2). However, the composition differed between petunia and calibrachoa: the ratios of (9Z)-violaxanthin and (all-ɛ)-β-carotene were higher in calibrachoa, and (all-ɛ)-lutein and (all-ɛ)-antheraxanthin + (all-ɛ)-zeaxanthin were higher in petunia (Table 1).

HPLC analysis of carotenoids

An acetone extract of frozen sample (0.1 g) was partitioned between diethyl ether and aqueous NaCl. The organic layer was washed with 5 mM Tris-HCl (pH 8.0) and was divided into two portions. One portion was saponified with an equivalent volume of 5% KOH/MeOH (w/v) for 1 h at room temperature; the other was dried and dissolved in 10% methyl tert-butyl ether (MTBE)/MeOH (v/v) (non-saponified sample). The KOH-treated sample was extracted with diethyl ether and washed with water. The organic layer was dried and dissolved in 10% MTBE/MeOH (saponified sample). Samples were analyzed by HPLC as described by Liu et al. (2013). Peaks were identified by comparing the retention times and absorbance spectra with those of carotenoids previously identified in the petals of chrysanthemum (Kishimoto et al. 2004) and tomato (Ariizumi et al. 2014). The contents of individual and total carotenoids were estimated from the peak areas of the HPLC chromatograms. The contents of esterified xanthophylls in tubes and limbs were calculated from the difference in peak areas between saponified and non-saponified samples. Measurements were performed in biological triplicate. Statistically significant differences were determined by Tukey’s test at the 5% level.

Comparison of carotenoid metabolic gene expression between pale-yellow-flowered petunia and deep-yellow-flowered calibrachoa

We compared the expressions of Actin between petunia and calibrachoa (Supplemental Fig. 1). Although the expression levels were different among tissues in both petunia and calibrachoa, they showed similar levels when we compared the same tissue in both plants. Similarities in cDNA sequences encoding each gene were very high (>90%) for both the plants (Supplemental Table 4). Therefore, we compared the expressions of each gene between petunia and calibrachoa with the same primers for RT-qPCR and conducted subsequent analyses.

Among the 18 carotenoid biosynthetic genes tested, expression levels of DXS, PDS, CRTISO, and CHYB/CYP97A in tubes and limbs were significantly higher in petunia than in calibrachoa (Fig. 4). In contrast, those of PI, PSY1, PSY2, LCYB, CHYB/CYP97C, ZEP, and XES in tubes and limbs were significantly lower in petunia than in calibrachoa.
Especially, LCYB and ZEP expression in petunia was extremely lower than that in calibrachoa. There was no clear correlation between carotenoid content and expression levels of GGPS1, Z-ISO, ZDS, LCYE, CHYB1, and CHYB2 (Fig. 4). We analyzed GGPS2 expression in calibrachoa using primers for petunia GGPS2 because we could not isolate GGPS2 cDNA from calibrachoa, but we could not detect GGPS2 expression in calibrachoa; this result suggests either that there is no ortholog of PhGGPS2 in calibrachoa or that the RT-qPCR primers did not match the calibrachoa GGPS2.

Among the 4 carotenoid cleavage genes tested, the expression of NCED in tubes and limbs was significantly higher and that of CCD1 in tubes and limbs was significantly lower in petunia than in calibrachoa, and CCD4a in petunia and CCD4b in calibrachoa were not detected (Fig. 4). We previously reported that CCD4a was expressed in white-flowered petunia cultivars but not in pale-yellow-flowered cultivars because of genomic insertions in the CCD4a promoter and coding regions (Kishimoto et al. 2018). We could not isolate CCD4b cDNA from calibrachoa and instead used the primers for PhCCD4b. It is possible that there is no ortholog of PhCCD4b in calibrachoa or that the RT-qPCR primers did not match the calibrachoa CCD4b.

**Similarity of amino acid sequences among PhXES, ChXES, and SlPYP1**

We isolated full-length cDNAs encoding XES from corollas of petunia (PhXES) and calibrachoa (ChXES). The deduced amino acid sequences of PhXES and ChXES consisted of 710 amino acids (Supplemental Fig. 2), showing 86% similarity. Similarity between PhXES and SIXES was 81% and that between ChXES and SIXES was 77%. BLAST searches against protein databases indicated that the polypeptide of PhXES and ChXES include α/β hydrolase fold domain (amino acids 132–383 in PhXES) and lysophospholipid acyltransferase (LPAT)-like domain (amino acids 132–383 in PhXES).
Table 1. Carotenoid contents and compositions in tubes, limbs, and leaves of petunia and calibrachoa

| Species                  | Tissue | (all-\(E\))-Neoxanthin (μg/g f.w.) | (all-\(E\))-Violaxanthin (μg/g f.w.) | (9′Z)-Neoxanthin (μg/g f.w.) | (9Z)-Violaxanthin (μg/g f.w.) | Unknown xanthophyll (μg/g f.w.) | (all-\(E\))-Lutein (μg/g f.w.) | (all-\(E\))-Zeaxanthin + (all-\(E\))-antheraxanthin (μg/g f.w.) | Unidentified (μg/g f.w.) | Total carotenoid content (μg/g f.w.) |
|-------------------------|--------|-----------------------------------|-------------------------------------|-------------------------------|-------------------------------|--------------------------------|-------------------------------|---------------------------------------------------------------|--------------------------|-----------------------------------|
| **Petunia “California Girl”** | Tube   | 3.59 ± 0.49                      | 4.29 ± 0.51                        | 2.95 ± 0.07                   | 4.11 ± 0.21                   | 2.93 ± 0.38                    | 6.82 ± 0.51                   | 3.36 ± 0.58                                                    | 5.78 ± 0.20              | 6.18 ± 0.50                        |
|                         |        | 8.96 ± 1.24                      | 10.73 ± 1.27                      | 7.38 ± 0.19                   | 10.27 ± 0.53                  | 7.33 ± 0.96                    | 17.05 ± 1.26                  | 8.41 ± 1.45                                                    | 14.4 ± 0.50              | 15.44 ± 1.24                      |
|                         | (%)    | 8.96 ± 1.24                      | 10.73 ± 1.27                      | 7.38 ± 0.19                   | 10.27 ± 0.53                  | 7.33 ± 0.96                    | 17.05 ± 1.26                  | 8.41 ± 1.45                                                    | 14.4 ± 0.50              | 15.44 ± 1.24                      |
|                         | Limb   | 1.23 ± 0.24                      | 2.11 ± 0.51                       | 1.70 ± 0.05                   | 1.49 ± 0.15                   | 2.37 ± 0.04                    | 6.29 ± 0.27                    | 3.94 ± 0.88                                                    | 3.98 ± 0.40              | 2.54 ± 0.13                        |
|                         | (%)    | 4.83 ± 0.95                      | 8.22 ± 2.01                      | 6.62 ± 0.20                   | 5.81 ± 0.57                   | 9.23 ± 0.15                    | 27.54 ± 1.04                  | 15.35 ± 3.42                                                   | 15.5 ± 1.57              | 9.90 ± 0.52                        |
|                         | Leaf   | 1.64 ± 0.45                      | 9.31 ± 2.13                       | 9.26 ± 0.62                   | 9.16 ± 0.31                   | 0.31 ± 0.31                    | 51.33 ± 1.26                  | 1.23 ± 0.27                                                    | 12.9 ± 0.15              | 12.60 ± 0.76                        |
|                         | (%)    | 1.63 ± 0.45                      | 9.29 ± 2.13                       | 9.24 ± 0.62                   | 9.16 ± 0.31                   | 0.31 ± 0.31                    | 51.21 ± 1.25                  | 1.23 ± 0.27                                                    | 12.9 ± 0.15              | 12.57 ± 0.75                        |
| **Calibrachoa “Tifosi Deep Yellow”** | Tube   | 17.64 ± 1.29                     | 31.14 ± 1.51                      | 30.40 ± 4.15                   | 41.78 ± 2.75                   | 12.75 ± 0.30                   | 23.62 ± 1.89                  | 13.46 ± 1.55                                                    | 61.18 ± 10.01            | 45.30 ± 4.64                       |
|                         | (%)    | 17.64 ± 1.29                     | 31.14 ± 1.51                      | 30.40 ± 4.15                   | 41.78 ± 2.75                   | 12.75 ± 0.30                   | 23.62 ± 1.89                  | 13.46 ± 1.55                                                    | 61.18 ± 10.01            | 45.30 ± 4.64                       |
|                         | Limb   | 13.23 ± 1.42                     | 41.87 ± 4.72                      | 20.50 ± 2.53                   | 29.38 ± 4.31                   | 12.80 ± 1.32                   | 29.89 ± 3.40                  | 10.40 ± 1.22                                                    | 67.86 ± 5.28             | 57.37 ± 4.50                       |
|                         | (%)    | 16.36 ± 0.47                      | 31.14 ± 1.51                      | 30.40 ± 4.15                   | 41.78 ± 2.75                   | 12.75 ± 0.30                   | 23.62 ± 1.89                  | 13.46 ± 1.55                                                    | 61.18 ± 10.01            | 45.30 ± 4.64                       |
|                         | Leaf   | 5.47 ± 0.11                      | 13.66 ± 1.18                      | 13.84 ± 1.68                   | 7.28 ± 0.27                   | 4.82 ± 0.32                    | 79.00 ± 8.09                  | 6.19 ± 0.54                                                    | 25.08 ± 1.00             | 24.43 ± 2.74                       |
|                         | (%)    | 3.21 ± 0.06                      | 8.01 ± 0.69                       | 8.12 ± 0.98                   | 1.63 ± 0.16                   | 2.83 ± 0.19                    | 46.35 ± 4.74                  | 3.63 ± 0.32                                                    | 14.71 ± 0.59             | 14.33 ± 1.60                       |

a Ratio of carotenoid component to total carotenoids (%).

b Means ± SE (n = 3).

c The same letter within a component indicates no significant difference by Tukey’s test (\(P < 0.05\)).
Fig. 3. Contents and ratios of esterified xanthophylls in tubes, limbs, and leaves of petunia and calibrachoa. all-E-neo, (all-Ε-neoxanthin; all-E-vio, (all-Ε)-violaxanthin; 9′Z-neo, (9′Z-neoxanthin; 9Z-vio, (9Z)-violaxanthin; unk, unknown xanthophyll; lut, (all-Ε)-lutein; zea + ant, (all-Ε)-zeaxanthin and (all-Ε)-antheraxanthin; β-car, (all-Ε)-β-carotene; unidentified, unidentified carotenoids. Analyses were performed in triplicate; means ± SE are shown. The same letter within a component indicates no significant difference by Tukey’s test ($P < 0.05$).

Fig. 4. Expression analysis of carotenoid metabolic genes in tubes, limbs, and leaves of petunia and calibrachoa. RT-qPCR analyses were performed in triplicate; means ± SE are shown. The same letter within a gene indicates no significant difference by Tukey’s test ($P < 0.05$).
Carotenoids in petunia and calibrachoa

Acids 407-679 in PhXES). Both XES contained chloroplast transit peptides (cTPs) at the N-terminus, indicating that they might be transported to the chloroplast. The predicted lengths of the cTPs were 29 amino acids in both PhXES and ChXES.

### Discussion

Although a wide range of flower colors has been developed in petunia cultivars, there are no deep-yellow-flowered cultivars. In corollas of both petunia and calibrachoa, eight kinds of xanthophylls including two kinds of 9-cis isomers and β-carotene were mainly detected. These carotenoids had similar absorption maxima (Britten 1995). Therefore, the corolla color tone of petunia and calibrachoa might be determined not by the ratio of carotenoid components but by the total accumulation level. To find out why petunia corollas cannot accumulate enough carotenoids to express deep-yellow color, we compared carotenogenic gene expressions between pale-yellow-flowered petunia and deep-yellow-flowered calibrachoa. We found several genes whose expression levels differed significantly between them (Figs. 4, 5).

Among biosynthetic genes tested, PSY1, PSY2, LCYB, and LCYE had significantly lower expression in petunia than in calibrachoa. PSY is a key enzyme of carotenoid biosynthesis functioning upstream in the pathway (Fig. 5; Cazzonelli and Pogson 2010, Ohmiya 2013). A low carotenoid content in petals is attributed to low PSY expression in carnation (Ohmiya et al. 2013), eustoma (Liu et al. 2013), Japanese morning glory (Yamamizo et al. 2010), and marigold (Moehs et al. 2001). We reported previously that PSY1 expression in petunia differed significantly between white-flowered and pale-yellow-flowered cultivars, but PSY2 expression did not (Kishimoto et al. 2018). Here, we showed that expression of both PSY1 and PSY2 was significantly lower in petunia than in calibrachoa. Therefore, low PSY expression is likely to be one of the causes of low carotenoid content in corollas of petunia.

LCYB and LCYE catalyze formation of β- and ε-rings, respectively (Fig. 5; Cunningham et al. 1996, Hugueney et al. 1995). The balance between LCYB and LCYE activities determines the ratio of β,β-carotenoids (β-carotene and its derivatives) to β,ε-carotenoids (α-carotene and its derivatives) (Cunningham et al. 1996, Ohmiya 2013). In petals of Oncidium (Chiou et al. 2010) and tubers of potato (Diretto et al. 2006), higher expression of LCYB than of LCYE causes the higher accumulation of β,β-carotenoids. In contrast, in petals of marigold (Moehs et al. 2001) and chrysanthemum (Kishimoto and Ohmiya 2006), higher expression of LCYE than of LCYB causes the higher accumulation of β,ε-carotenoids. In tomato, the expression of both LCYB and LCYE is decreased during fruit development, resulting in the low accumulation of cyclic carotenoids and the high accumulation of lycopene (Ronan et al. 1999). In corollas of petunia, the expression of both LCYB and LCYE was significantly lower than in calibrachoa, but lycopene was undetectable (Figs. 2, 4). Plant species accumulating lycopene in their petals are very rare (Ohmiya 2011). It is likely that most species, including petunia, are unable to accumulate lycopene in petals. Therefore, it is possible that lack of ability to accumulate lycopene and the low level of lycopene cyclization activity in petunia cause not only low accumulation of cyclic carotenoids, but also low levels of total carotenoids.

Carotenoid degradation is another factor that affects carotenoid accumulation in flowers. We examined the expression of carotenoid catabolic genes to see whether carotenoid

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**Fig. 5.** Putative carotenoid biosynthetic pathway in corollas of petunia. GA3P, glyceraldehyde 3-phosphate; DXS, 1-deoxy-d-xylulose 5-phosphate synthase; DOXP, 1-deoxy-d-xylulose 5-phosphate; MEP, 2-C-methyl-d-erythritol-2,4-cyclodiphosphate; IPP, isopentenyl diphosphate; IP1, IPP isomerase; GGPS, GGPP synthase; GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-cis-ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCYB, lycopene β-ring cyclase; LCYE, lycopene ε-ring cyclase; CHYB, β-ring hydroxylase; CHYE, ε-ring hydroxylase; CHYB/CHYB2, cytochrome P450-type β-ring hydroxylase; CHYE/CHYE2, cytochrome P450-type ε-ring hydroxylase; ZEP, zeaxanthin epoxidase; NCED, 9-cis-epoxy carotenoid dioxygenase; ABA, abscisic acid; CCD1, carotenoid cleavage dioxygenase 1; CCD4a, carotenoid cleavage dioxygenase 4a; XES, xanthophyll esterase. Major carotenoids accumulated in corollas of petunia and calibrachoa are indicated in bold letters. Thickness of arrows indicates the level of gene expression in tubes relative to calibrachoa.
degradation is involved in the regulation of carotenoid accumulation in petunia corollas. The expression of NCED was significantly higher in petunia than in calibrachoa (Fig. 4). NCED cleaves (9′Z)-neoxanthin and (9Z)-violaxanthin to xanthoxin, an intermediate in the biosynthesis of abscisic acid (Fig. 5; Schwartz et al. 1997). Kato et al. (2004, 2006) found an inverse relationship between CitNCED2 expression and (9Z)-violaxanthin level in citrus fruits. In corollas of petunia, the low levels of (9′Z)-neoxanthin and (9Z)-violaxanthin might be due to the high NCED expression.

There was no inverse relationship of CCD1 or CCD4a expression with carotenoid content in calibrachoa or petunia. CCD1 cleaves carotenoids at 9,10 (9′,10′) double bonds and contributes to the emission of β- and α-ionones, important fragrance components, in flowers of petunia and Osmanthus fragrans (Baldermann et al. 2010, Simkin et al. 2004b). However, lack of correlation between CCD1 expression and carotenoid content has been demonstrated in petals of Japanese morning glory (Yamamizo et al. 2010), rice endosperm (Ilg et al. 2010), tomato fruit (Simkin et al. 2004a), and citrus fruit (Kato et al. 2006), possibly because CCD1 is located in the cytoplasm and has limited access to its substrates in chromoplasts (Bouvier et al. 2003, McCarty and Klee 2006). We previously reported that CCD1 is constitutively expressed in corollas of both white-flowered and pale-yellow-flowered cultivars (Kishimoto et al. 2018). Therefore, we consider that the expression of CCD1 did not affect the carotenoid content in corollas of petunia and calibrachoa. Ohmiya et al. (2006) revealed that CCD4 plays a key role in cleavage of carotenoids in petals of chrysanthemum. There is increasing evidence to show that CCD4 is involved in the regulation of carotenoid accumulation in chromoplasts of flowers and fruits (Falchi et al. 2013, Gonzalez-Jorge et al. 2013, Hai et al. 2012, Zhang et al. 2015). We have previously shown that an insertion in the putative promoter region and a palindromic sequence in the coding region of the CCD4a genomic sequence of pale-yellow petunia prevents CCD4a expression (Kishimoto et al. 2018). In contrast, white petunia corollas have high CCD4a expression. Therefore, CCD4a activity is predicted to be a major cause of extremely low levels of carotenoids in white-flowered petunia cultivars. Although calibrachoa had substantial CCD4a expression both in tubes and limbs, we expect that biosynthesis greatly exceeds degradation. Therefore, CCD4a is not a key determinant of carotenoid accumulation in calibrachoa corolla. CCD4b expression was detected only in petunia. However, we speculate that CCD4b is not involved in the regulation of carotenoid accumulation in corollas of petunia, because the expression pattern of CCD4b in petunia was not associated with carotenoid content (Kishimoto et al. 2018).

Esterification of xanthophylls is important for mass accumulation of carotenoids in chromoplasts. Ariizumi et al. (2014) showed that disruption of PYP1 (encoding XES) causes not only loss of esterified xanthophylls, but also a drastic decrease in total carotenoid levels in tomato petals. We showed that the ratio of esterified to total xanthophylls was significantly lower in petunia than in calibrachoa (Fig. 1E). We assume that esterification activity is lower in petunia than in calibrachoa because of lower XES expression (Fig. 4). Both cis- and trans-forms of neoxanthin and violaxanthin were almost completely esterified in calibrachoa, but were incompletely esterified in petunia; in particular, ratios of esterification of trans-forms of neoxanthin and violaxanthin were <50% (Fig. 3). These results suggest that petunia XES has higher substrate specificity for cis-xanthophylls than for trans-xanthophylls. It is possible that the substrate specificity in PhXES is due to the amino acids divergence in the conserved domain important for esterase/lipase activity (Supplemental Fig. 2). However, the contents of cis-xanthophylls, such as (9′Z)-neoxanthin and (9Z)-violaxanthin, were low in all pale-yellow-flowered petunia cultivars tested (Kishimoto et al. 2018). The expression of ZEP, which catalyzes epoxidation of zeaxanthin to produce violaxanthin (Fig. 5; Marin et al. 1996), was significantly lower in petunia corollas than in calibrachoa corollas (Fig. 4); therefore, one reason for the low level of cis-forms of neoxanthin and violaxanthin in petunia (Table 1) would be low epoxidation activity. In addition, cleavage activity of NCED to cis-forms of neoxanthin and violaxanthin is likely to be higher in petunia than in calibrachoa, as mentioned above. Recently, Ma et al. (2017) reported that citrus CCD1 and CCD4 can cleave free but not esterified β-cryptoxanthin in vitro. The higher ratio of free forms of xanthophylls in petunia leads us to speculate that carotenoids in petunia corollas are more susceptible to cleavage by CCD family enzymes.

We conclude that low carotenoid accumulation in corollas of petunia is due to low biosynthesis of violaxanthin, high cleavage activity, especially cleavage of violaxanthin and neoxanthin, and low ratios of esterified xanthophylls. We assume that low expression of XES and low substrate specificity of XES for trans-xanthophylls in petunia result in a low ratio of esterification of xanthophylls. The mechanism by which esterification of xanthophylls promotes carotenoid accumulation remains unclear; therefore, further studies will be needed to clarify how.

Acknowledgments

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