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Research Paper

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.

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*)

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mutants of tomato. They showed that disruption of *PYP1* caused complete loss of esterified xanthophylls, reduction in total carotenoid content, and abnormal chromoplast development in petals. The results suggest that esterification is important for mass accumulation of carotenoids in petals.

Petunia (*Petunia hybrida*) is an important ornamental plant and an excellent model for investigating floral pigmentation (Tornielli *et al.* 2008), which ranges wide from pink to red to purple, due mainly to anthocyanins (Muszyński 1964). Several pale-yellow-flowered petunia cultivars are now available, but no deep-yellow cultivars exist. The main pigments in the corollas of the pale-yellow cultivars are carotenoids, albeit at a lower content than in deep-yellow flowers of other species (Kishimoto *et al.* 2018, Murakami *et al.* 2003, Nielsen and Bloor 1997). We previously reported that carotenoid accumulation in pale-yellow petunia corollas was due to higher expression of several biosynthetic genes than in white cultivars and lower catabolic activity due to complete lack of *CCD4a* expression (Kishimoto *et al.* 2018).

Here, to find why petunia does not accumulate high amounts of carotenoids in its corollas, we compared carotenoid composition and metabolic gene expression between pale-yellow-flowered petunia and deep-yellow-flowered calibrachoa (*Calibrachoa hybrida*), a close relative (Jędrzejuk *et al.* 2017, Olmstead *et al.* 2008), whose corolla color is derived from carotenoids (Murakami *et al.* 2004). 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-*

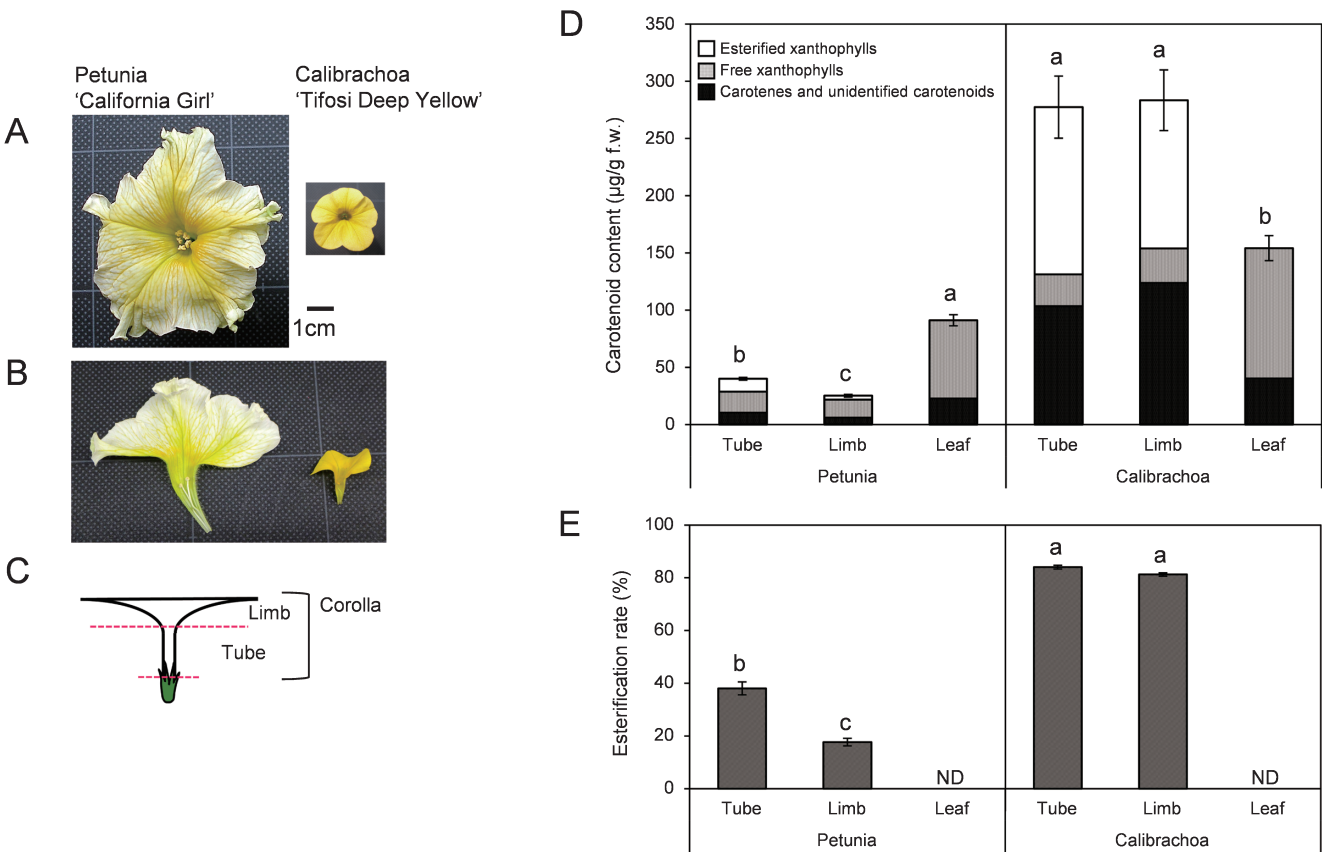

Fig. 1. (A) Flowers of petunia 'California Girl' and calibrachoa 'Tifosi Deep Yellow'. (B) Vertical sections of corollas. (C) Sampling positions of tube and limb. (D) Carotenoid content in tubes, limbs, and leaves of petunia and calibrachoa. (E) Ratio of esterified xanthophylls to total xanthophylls.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

D-xylulose 5-phosphate synthase (*ChDXS*), LC335780; *isopentenyl diphosphate isomerase* (*ChIPI*), LC335781; *geranylgeranyl pyrophosphate synthase* (*ChGGPS1*), LC335782; *phytoene synthase 1* (*ChPSY1*), LC335783; *ChPSY2*, LC335784; *phytoene desaturase* (*ChPDS*), LC335785; *15*-cis-*ζ-carotene isomerase* (*ChZ-ISO*), LC369596; *ζ-carotene desaturase* (*ChZDS*), LC335786; *carotenoid isomerase* (*ChCRTISO*), LC369595; *lycopene β-ring cyclase* (*ChLCYB*), LC335787; *lycopene ε-ring cyclase* (*ChLCYE*), LC335788; *β-ring hydroxylase 1* (*ChCHYB1*), LC335789; *ChCHYB2*, LC335790; *cytochrome P450-type β-ring hydroxylase* (*ChCHYB/CYP97A*), LC361460; *cytochrome P450-type ε-ring hydroxylase* (*ChCHYE/CYP97C*), LC361461; *zeaxanthin epoxygenase* (*ChZEP*), LC335791; *ChXES*, LC335779; *carotenoid cleavage dioxygenase 1* (*ChCCD1*), LC335792; *carotenoid cleavage dioxygenase 4* (*ChCCD4a*), LC335793; *9*-cis-*epoxycarotenoid dioxygenase* (*ChNCED*), LC335794; *ChActin*, LC335795, *PhXES*, LC335778. Other cDNA sequences of petunia were previously reported (Kishimoto *et al.* 2018). Similarity of cDNA sequences encoding each gene between petunia and calibrachoa and putative amino acid sequences among PhXES, ChXES, and SlPYP1 were analyzed by clustalW (Larkin *et al.* 2007). Prediction of the presence of chloroplast/chromoplast transit peptides (cTP) in the protein sequences and the location of potential cTP cleavage sites were carried out using the ChloroP 1.1 server (Emanuelsson *et al.* 1999).

Quantitative real-time PCR analysis

Total RNA was isolated with Trizol reagent in a PureLink RNA mini column (both Thermo Fisher Scientific). Reverse-transcription quantitative real-time PCR (RT-qPCR) was performed as described by Kishimoto *et al.* (2018). Primers for carotenoid metabolic genes were designed from sequences of partial-length cDNAs conserved between petunia and calibrachoa in Oligo software (**Supplemental Table 3**). RT-qPCR analyses were performed in technical triplicate. Statistically significant differences were determined by Tukey's test at the 5% level.

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.

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-*E*)-neoxanthin, (all-*E*)-violaxanthin, (9′*Z*)-neoxanthin, (9*Z*)-violaxanthin, (all-*E*)-lutein, (all-*E*)-zeaxanthin, (all-*E*)-antheraxanthin, and (all-*E*)-β-carotene (**Fig. 2**). However, the composition differed between petunia and calibrachoa: the ratios of (9*Z*)-violaxanthin and (all-*E*)-β-carotene were higher in calibrachoa, and (all-*E*)-lutein and (all-*E*)-antheraxanthin + (all-*E*)-zeaxanthin were higher in petunia (**Table 1**).

HPLC analysis detected esterified xanthophylls in the non-saponified samples at a retention time of approximately 20 to 30 min in all limbs and tubes tested but not in leaves (**Fig. 2**). Ratios of esterified to total xanthophylls were >80% in calibrachoa, but <40% in petunia (**Fig. 1E**). The ratio of esterification of each xanthophyll also differed between species (**Fig. 3**): in calibrachoa, those of all xanthophylls except for lutein were >70%, but in petunia, those of most xanthophylls except for (9′*Z*)-neoxanthin in tubes and (9*Z*)-violaxanthin in tubes and limbs were <50%.

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 *IPI*, *PSY1*, *PSY2*, *LCYB*, *CHYE/CYP97C*, *ZEP*, and *XES* in tubes and limbs were significantly lower in petunia than in calibrachoa.

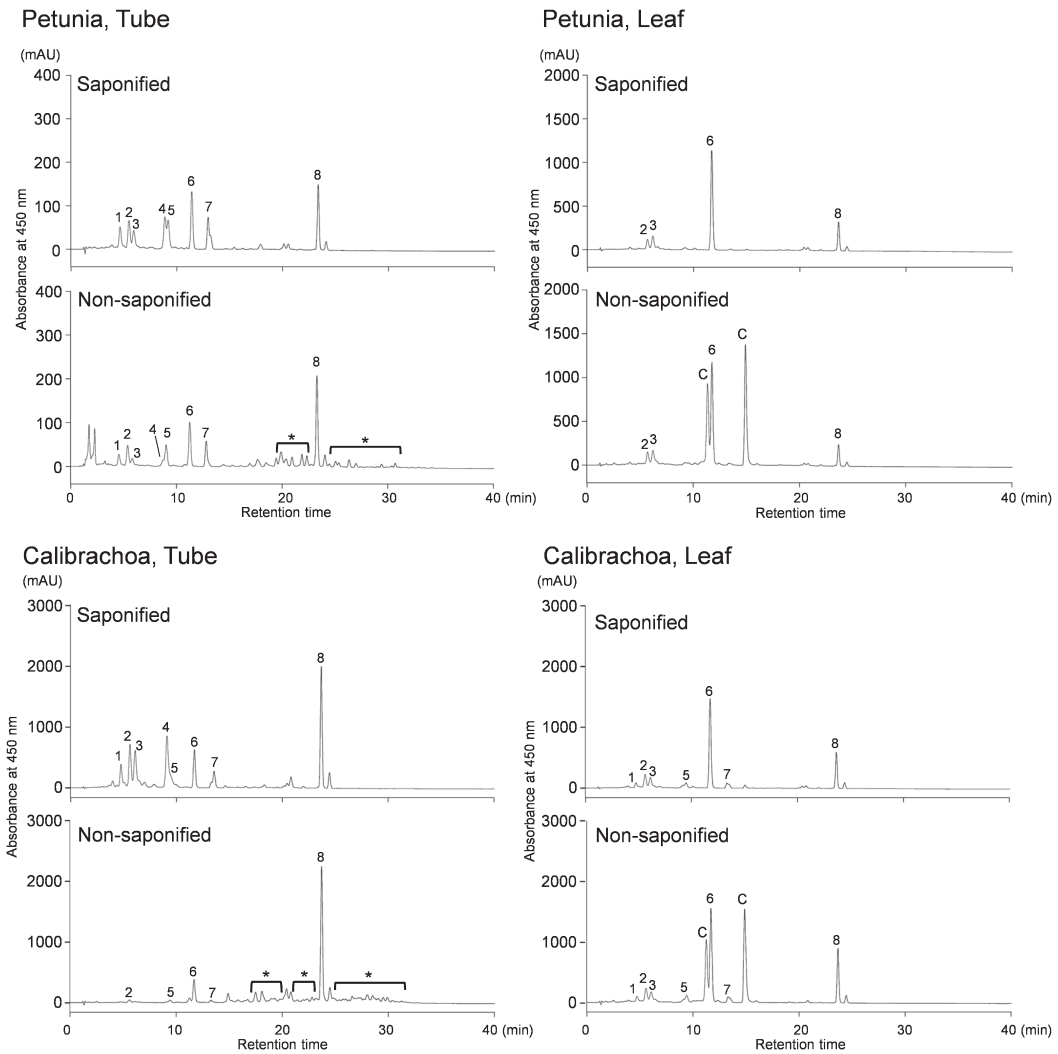

Fig. 2. HPLC chromatograms of carotenoid extracts from tubes and leaves of petunia and calibrachoa. Saponified and non-saponified carotenoid extracts from 0.025 g f.w. of tubes and leaves were analyzed. Peaks: 1, (all-*E*)-neoxanthin; 2, (all-*E*)-violaxanthin; 3, (9′*Z*)-neoxanthin; 4, (9*Z*)-violaxanthin; 5, unknown xanthophyll; 6, (all-*E*)-lutein; 7, (all-*E*)-antheraxanthin + (all-*E*)-zeaxanthin; 8, (all-*E*)-β-carotene; C, chlorophylls. *Esterified xanthophylls.

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 SlXES was 81% and that between ChXES and SlXES 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

Table 1. Carotenoid contents and compositions in tubes, limbs, and leaves of petunia and calibrachoa

Species	Tissue	Carotenoid component									Total carotenoid content (μg/g f.w.)
		(all-*E*)-Neoxanthin	(all-*E*)-Violaxanthin	(9′*Z*)-Neoxanthin	(9*Z*)-Violaxanthin	Unknown xanthophyll	(all-*E*)-Lutein	(all-*E*)-Zeaxanthin + (all-*E*)-antheraxanthin	(all-*E*)-β-Carotene	Unidentified	
Petunia "California Girl"	Tube (μg/g f.w.)	3.59 ± 0.49[b]	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	40.01 ± 1.20 cd
	(%)[a]	8.96 ± 1.24 a[c]	10.73 ± 1.27 a	7.38 ± 0.19 ab	10.27 ± 0.53 b	7.33 ± 0.96 a	17.05 ± 1.26 bc	8.41 ± 1.45 ab	14.4 ± 0.50 c	15.44 ± 1.24 abc	
	Limb (μg/g f.w.)	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	25.65 ± 0.90 d
	(%)	4.83 ± 0.95 b	8.22 ± 2.01 a	6.62 ± 0.20 b	5.81 ± 0.57 c	9.23 ± 0.15 a	27.54 ± 1.04 b	15.35 ± 3.42 a	15.5 ± 1.57 abc	9.90 ± 0.52 c	
	Leaf (μg/g f.w.)	1.64 ± 0.45	9.31 ± 2.13	9.26 ± 0.62	1.96 ± 0.31	0.31 ± 0.31	51.33 ± 1.26	1.23 ± 0.27	12.9 ± 0.15	12.60 ± 0.76	100.23 ± 5.40 bc
	(%)	1.63 ± 0.45 bc	9.29 ± 2.13 a	9.24 ± 0.62 ab	1.96 ± 0.31 cd	0.31 ± 0.31 c	51.21 ± 1.25 a	1.23 ± 0.27 b	12.9 ± 0.15 c	12.57 ± 0.75 bc	
Calibrachoa "Tifosi Deep Yellow"	Tube (μg/g f.w.)	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	277.29 ± 27.13 a
	(%)	6.36 ± 0.47 ab	11.23 ± 0.55 a	10.96 ± 1.50 a	15.07 ± 0.99 a	4.60 ± 0.11 b	8.52 ± 0.68 c	4.86 ± 0.56 b	22.07 ± 3.61 ab	16.34 ± 1.67 abc	
	Limb (μg/g f.w.)	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	283.31 ± 26.50 a
	(%)	4.67 ± 0.50 b	14.78 ± 1.67 a	7.24 ± 0.89 ab	10.37 ± 1.52 b	4.52 ± 0.47 b	10.55 ± 1.20 c	3.67 ± 0.43 b	23.95 ± 1.86 a	20.25 ± 1.59 a	
	Leaf (μg/g f.w.)	5.47 ± 0.11	13.66 ± 1.18	13.84 ± 1.68	2.78 ± 0.27	4.82 ± 0.32	79.00 ± 8.09	6.19 ± 0.54	25.08 ± 1.00	24.43 ± 2.74	170.45 ± 15.20 b
	(%)	3.21 ± 0.06 b	8.01 ± 0.69 a	8.12 ± 0.98 ab	1.63 ± 0.16 d	2.83 ± 0.19 b	46.35 ± 4.74 a	3.63 ± 0.32 b	14.71 ± 0.59 bc	14.33 ± 1.60 ab	

[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 ($P < 0.05$).

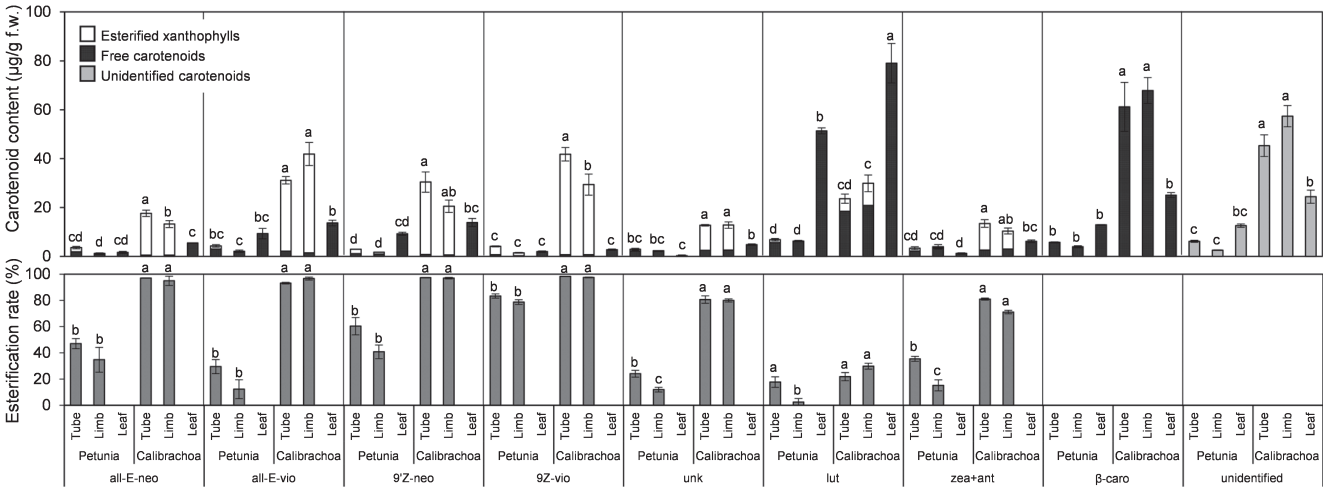

Fig. 3. Contents and ratios of esterified xanthophylls in tubes, limbs, and leaves of petunia and calibrachoa. all-E-neo, (all-*E*)-neoxanthin; all-E-vio, (all-*E*)-violaxanthin; 9′Z-neo, (9′*Z*)-neoxanthin; 9Z-vio, (9*Z*)-violaxanthin; unk, unknown xanthophyll; lut, (all-*E*)-lutein; zea + ant, (all-*E*)-zeaxanthin and (all-*E*)-antheraxanthin; β-caro, (all-*E*)-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 ($P < 0.05$).

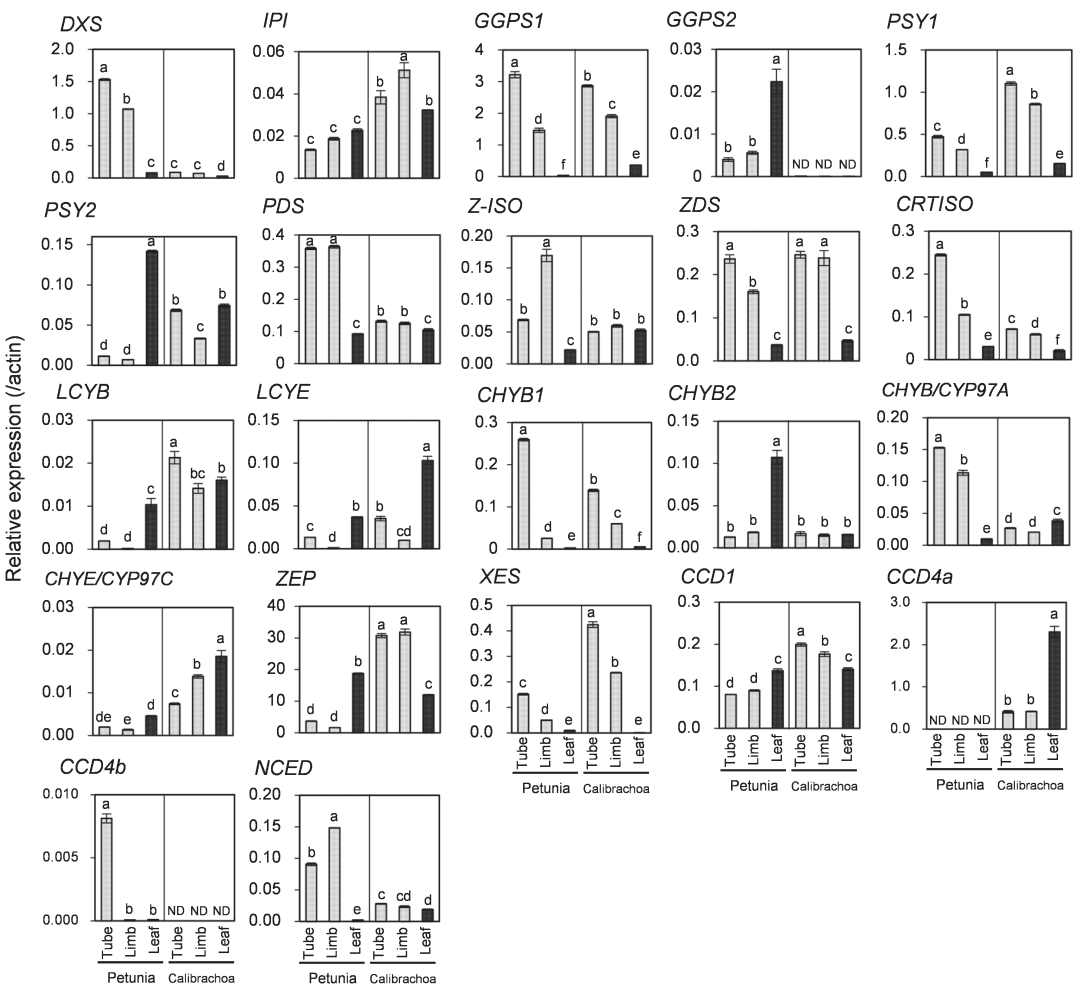

Fig. 4. Expression analysis of carotenoid metabolic genes in tubes, limbs, and leaves of petunia and calibrachoa. RT-qPCR analyses were performed in triplicate; means ± SE are shown. The same letter within a gene indicates no significant difference by Tukey's test ($P < 0.05$).

acids 407–679 in PhXES). Both XES contained chloroplast transit peptides (cTPs) at the N-terminus, indicating that they might be transported to the chromoplast. 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 (Ronen *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

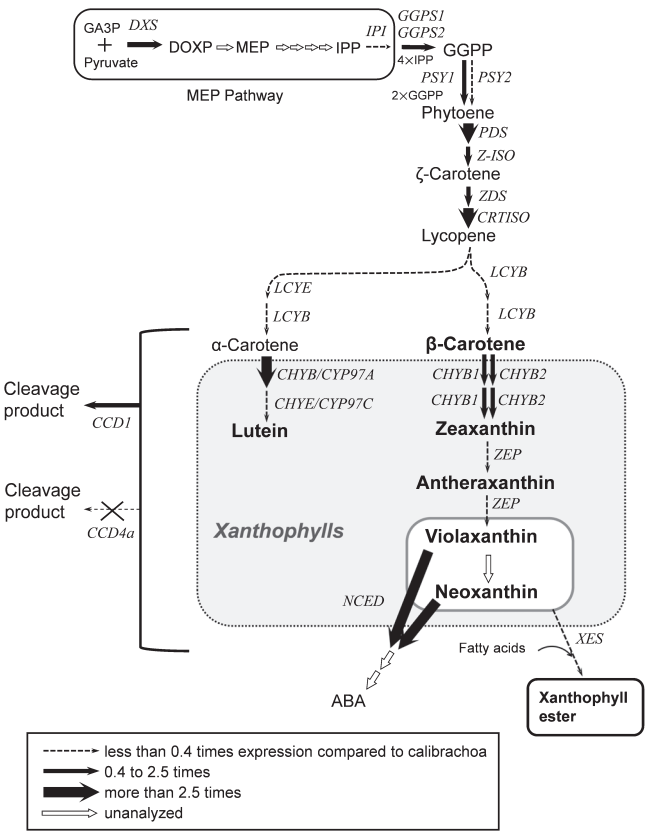

Fig. 5. Putative carotenoid biosynthetic pathway in corollas of petunia. GA3P, glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DOXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol-2,4-cyclodisphosphate; IPP, isopentenyl diphosphate; IPI, IPP isomerase; GGPS, GGPP 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/CYP97A, cytochrome P450-type β-ring hydroxylase; CHYE/CYP97C, 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 (9*Z*)-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 (9*Z*)-violaxanthin level in citrus fruits. In corollas of petunia, the low levels of (9′*Z*)-neoxanthin and (9*Z*)-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 (9*Z*)-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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Literature Cited

Ariizumi, T., S. Kishimoto, R. Kakami, T. Maoka, H. Hirakawa, Y. Suzuki, Y. Ozeki, K. Shirasawa, S. Bernillon, Y. Okabe *et al.* (2014) Identification of the carotenoid modifying gene *PALE YELLOW PETAL 1* as an essential factor in xanthophyll esterification and yellow flower pigmentation in tomato (*Solanum lycopersicum*). Plant J. 79: 453–465.

Baldermann, S., M. Kato, M. Kurosawa, Y. Kurobayashi, A. Fujita, P. Fleischmann and N. Watanabe (2010) Functional characterization

of a carotenoid cleavage dioxygenase 1 and its relation to the carotenoid accumulation and volatile emission during the floral development of *Osmanthus fragrans* Lour. J. Exp. Bot. 61: 2967–2977.

Bouvier, F., C. Suire, J. Mutterer and B. Camara (2003) Oxidative remodeling of chromoplast carotenoids: identification of the carotenoid dioxygenase *CsCCD* and *CsZCD* genes involved in crocus secondary metabolite biogenesis. Plant Cell 15: 47–62.

Breithaupt, D.E., U. Wirt and A. Bamedi (2002) Differentiation between lutein monoester regioisomers and detection of lutein diesters from marigold flowers (*Tagetes erecta* L.) and several fruits by liquid chromatography−mass spectrometry. J. Agric. Food Chem. 50: 66–70.

Britton, G. (1995) UV/Visible Spectroscopy. *In*: Britton, G., S. Liaaen-Jensen and H. Pfander (eds.) Carotenoids Volume 1B: Spectroscopy, Birkhäuser, Basel, Boston, Berlin, pp. 13–62.

Camara, B., P. Hugueney, F. Bouvier, M. Kuntz and R. Monéger (1995) Biochemistry and molecular biology of chromoplast development. Int. Rev. Cytol. 163: 175–247.

Cazzonelli, C.I. and B.J. Pogson (2010) Source to sink: regulation of carotenoid biosynthesis in plants. Trends Plant. Sci. 15: 266–274.

Chiou, C.-Y., H.-A. Pan, Y.-N. Chuang and K.-W. Yeh (2010) Differential expression of carotenoid-related genes determines diversified carotenoid coloration in floral tissues of *Oncidium* cultivars. Planta 232: 937–948.

Cunningham, Jr., F.X., B. Pogson, Z. Sun, K.A. McDonald, D. DellaPenna and E. Gantt (1996) Functional analysis of the β and ε lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8: 1613–1626.

Deruère, J., S. Römer, A. d'Harlingue, R.A. Backhaus, M. Kuntz and B. Camara (1994) Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. Plant Cell 6: 119–133.

Diretto, G., R. Tavazza, R. Welsch, D. Pizzichini, F. Mourgues, V. Papacchioli, P. Beyer and G. Giuliano (2006) Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase. BMC Plant Biol. 6: 13.

Emanuelsson, O., H. Nielsen and G. von Heijne (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. 8: 978–984.

Falchi, R., E. Vendramin, L. Zanon, S. Scalabrin, G. Cipriani, I. Verde, G. Vizzotto and M. Morgante (2013) Three distinct mutational mechanisms acting on a single gene underpin the origin of yellow flesh in peach. Plant J. 76: 175–187.

Gonzalez-Jorge, S., S.H. Ha, M. Magallanes-Lundback, L.U. Gilliland, A. Zhou, A.E. Lipka, Y.N. Nguyen, R. Angelovici, H. Lin, J. Cepela *et al.* (2013) *CAROTENOID CLEAVAGE DIOXYGENASE4* is a negative regulator of β-carotene content in *Arabidopsis* seeds. Plant Cell 25: 4812–4826.

Goodwin, T.W. (1980) Carotenoids in higher plants. *In*: The Biochemistry of the Carotenoids Vol. 1 Plants. Chapman and Hall, London and New York, pp. 143–188.

Hai, M.T.L., J. Masuda, I. Miyajima, N.Q. Thien, N. Mojtahedi, M. Hiramatsu, J.-H. Kim and H. Okubo (2012) Involvement of *carotenoid cleavage dioxygenase 4* gene in tepal color change in *Lilium brownii* var. *colchesteri*. J. Japan. Soc. Hort. Sci. 81: 366–373.

Hirschberg, J. (2001) Carotenoid biosynthesis in flowering plants. Curr. Opin. Plant Biol. 4: 210–218.

Hugueney, P., A. Badillo, H.C. Chen, A. Klein, J. Hirschberg, B. Camara and M. Kuntz (1995) Metabolism of cyclic carotenoids: a model for the alteration of this biosynthetic pathway in *Capsicum annuum* chromoplasts. Plant J. 8: 417–424.

Ilg, A., Q. Yu, P. Schaub, P. Beyer and S. Al-Babili (2010) Overexpression of the *rice carotenoid cleavage dioxygenase 1* gene in Golden Rice endosperm suggests apocarotenoids as substrates *in planta*. Planta 232: 691–699.

Jędrzejuk, A., L. Meyer and M. Serek (2017) Characterization of interspecific hybrids of *Petunia* and *Calibrachoa* by multiplex PCR, DNA content, and chromosome number. J. Hortic. Sci. Biotechnol. 92: 493–501.

Kato, M., Y. Ikoma, H. Matsumoto, M. Sugiura, H. Hyodo and M. Yano (2004) Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiol. 134: 824–837.

Kato, M., H. Matsumoto, Y. Ikoma, H. Okuda and M. Yano (2006) The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation of citrus fruit. J. Exp. Bot. 57: 2153–2164.

Kishimoto, S., T. Maoka, M. Nakayama and A. Ohmiya (2004) Carotenoid composition in petals of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura). Phytochemistry 65: 2781–2787.

Kishimoto, S. and A. Ohmiya (2006) Regulation of carotenoid biosynthesis in petals and leaves of chrysanthemum (*Chrysanthemum morifolium*). Physiol. Plant. 128: 437–447.

Kishimoto, S., K. Sumitomo, M. Yagi, M. Nakayama and A. Ohmiya (2007) Three routes to orange petal colour via carotenoid component in 9 Compositae species. J. Japan. Soc. Hort. Sci. 76: 250–257.

Kishimoto, S., C. Oda-Yamamizo and A. Ohmiya (2018) Regulation of carotenoid pigmentation in corollas of petunia. Plant Mol. Biol. Rep. 36: 632–642.

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez *et al.* (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

Liu, H., S. Kishimoto, C. Yamamizo, N. Fukuta and A. Ohmiya (2013) Carotenoid accumulations and carotenogenic gene expressions in the petals of *Eustoma grandiflorum*. Plant Breed. 132: 417–422.

Ljubesić, N., M. Wrischer and Z. Devidé (1991) Chromoplasts–the last stages in plastid development. Int. J. Dev. Biol. 35: 251–258.

Ma, G., L. Zhang, K. Iida, Y. Madono, W. Yungyuen, M. Yahata, K. Yamawaki and M. Kato (2017) Identification and quantitative analysis of β-cryptoxanthin and β-citraurin esters in Satsuma mandarin fruit during the ripening process. Food Chem. 234: 356–364.

Maoka, T., T. Etoh, S. Kishimoto and S. Sakata (2011) Carotenoids and their fatty acid esters in the petals of *Adonis aestivalis*. J. Oleo. Sci. 60: 47–52.

Marin, E., L. Nussaume, A. Quesada, M. Gonneau, B. Sotta, P. Hugueney, A. Frey and A. Marion-Poll (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. EMBO J. 15: 2331–2342.

McCarty, D.R. and H.J. Klee (2006) Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. Plant J. 45: 982–993.

Moehs, C.P., L. Tian, K.W. Osteryoung and D. DellaPenna (2001) Analysis of carotenoid biosynthetic gene expression during marigold petal development. Plant Mol. Biol. 45: 281–293.

Murakami, Y., Y. Fukui, H. Watanabe, H. Kokubun, Y. Toya and T. Ando (2003) Distribution of carotenoids in the flower of non-yellow commercial petunia. J. Hortic. Sci. Biotechnol. 78: 127–130.

Murakami, Y., Y. Fukui, H. Watanabe, H. Kokubun, Y. Toya and T. Ando (2004) Floral coloration and pigmentation in *Calibrachoa* cultivars. J. Hortic. Sci. Biotechnol. 79: 47–53.

Muszyński, S. (1964) A survey of anthocyanidins in *Petunia*. Physiol. Plant. 17: 975–979.

Nielsen, K.M. and S.J. Bloor (1997) Analysis and developmental profile of carotenoid pigments in petals of three yellow petunia cultivars. Sci. Hortic. 71: 257–266.

Ohmiya, A., S. Kishimoto, R. Aida, S. Yoshioka and K. Sumitomo (2006) Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in chrysanthemum petals. Plant Physiol. 142: 1193–1201.

Ohmiya, A. (2009) Carotenoid cleavage dioxygenases and their apocarotenoid products in plants. Plant Biotechnol. 26: 351–358.

Ohmiya, A. (2011) Diversity of carotenoid composition in flower petals. Jpn. Agric. Res. Q. 45: 163–171.

Ohmiya, A. (2013) Qualitative and quantitative control of carotenoid accumulation in flower petals. Sci. Hortic. 163: 10–19.

Ohmiya, A., K. Tanase, M. Hirashima, C. Yamamizo and M. Yagi (2013) Analysis of carotenogenic gene expression in petals and leaves of carnation (*Dianthus caryophyllus* L.). Plant Breed. 132: 423–429.

Olmstead, R.G., L. Bohs, H.A. Migid, E. Santiago-Valentin, V.F. Garcia and S.M. Collier (2008) A molecular phylogeny of the Solanaceae. Taxon 57: 1159–1181.

Robert, B., P. Horton, A.A. Pascal and A.V. Ruban (2004) Insights into the molecular dynamics of plant light-harvesting proteins *in vivo*. Trends Plant Sci. 9: 385–390.

Ronen, G., M. Cohen, D. Zamir and J. Hirschberg (1999) Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant *Delta*. Plant J. 17: 341–351.

Ruban, A.V., R. Berera, C. Ilioaia, I.H.M. van Stokkum, J.T.M. Kennis, A.A. Pascal, H. van Amerongen, B. Robert, P. Horton and R. van Grondelle (2007) Identification of a mechanism of photoprotective energy dissipation in higher plants. Nature 450: 575–578.

Schwartz, S.H., B.C. Tan, D.A. Gage, J.A.D. Zeevaart and D.R. McCarty (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. Science 276: 1872–1874.

Simkin, A.J., S.H. Schwartz, M. Auldridge, M.G. Taylor and H.J. Klee (2004a) The tomato *carotenoid cleavage dioxygenase 1* genes contribute to the formation of the flavor volatiles β-ionone, pseudoionone, and geranylacetone. Plant J. 40: 882–892.

Simkin, A.J., B.A. Underwood, M. Auldridge, H.M. Loucas, K. Shibuya, E. Schmelz, D.G. Clark and H.J. Klee (2004b) Circadian regulation of the PhCCD1 carotenoid cleavage dioxygenase controls emission of β-ionone, a fragrance volatile of petunia flowers. Plant Physiol. 136: 3504–3514.

Tornielli, G., R. Koes and F. Quattrocchio (2008) The genetics of flower color. *In*: Gerats, T. and J. Strommer (eds.) Petunia: Evolutionary, Developmental and Physiological Genetics. Springer, New York, pp. 325–342.

Yamamizo, C., S. Kishimoto and A. Ohmiya (2010) Carotenoid composition and carotenogenic gene expression during *Ipomoea* petal development. J. Exp. Bot. 61: 709–719.

Zhang, B., C. Liu, Y. Wang, X. Yao, F. Wang, J. Wu, G.J. King and K. Liu (2015) Disruption of a *CAROTENOID CLEAVAGE DIOXYGENASE 4* gene converts flower colour from white to yellow in *Brassica* species. New Phytol. 206: 1513–1526.