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Carotenoid composition and carotenogenic gene expression during *Ipomoea* petal development

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Abstract

Japanese morning glory (*Ipomoea nil*) is a representative plant lacking a yellow-flowered cultivar, although a few wild *Ipomoea* species contain carotenoids in their petals such as *Ipomoea* sp. (yellow petals) and *I. obscura* (pale-yellow petals). In the present study, carotenoid composition and the expression patterns of carotenogenic genes during petal development were compared among *I. nil*, *I. obscura*, and *Ipomoea* sp. to identify the factors regulating carotenoid accumulation in *Ipomoea* plant petals. In the early stage, the carotenoid composition in petals of all the *Ipomoea* plants tested was the same as in the leaves mainly showing lutein, violaxanthin, and β-carotene (chloroplast-type carotenoids). However, in fully opened flowers, chloroplast-type carotenoids were entirely absent in *I. nil*, whereas they were present in trace amounts in the free form in *I. obscura*. At the late stage of petal development in *Ipomoea* sp., the majority of carotenoids were β-cryptoxanthin, zeaxanthin, and β-carotene (chromoplast-type carotenoids). In addition, most of them were present in the esterified form. Carotenogenic gene expression was notably lower in *I. nil* than in *Ipomoea* sp. In particular, β-ring hydroxylase (*CHYB*) was considerably suppressed in petals of both *I. nil* and *I. obscura*. The *CHYB* expression was found to be significantly high in the petals of *Ipomoea* sp. during the synthesis of chromoplast-type carotenoids. The expression levels of carotenoid cleavage genes (*CCD1* and *CCD4*) were not correlated with the amount of carotenoids in petals. These results suggest that both *I. obscura* and *I. nil* lack the ability to synthesize chromoplast-type carotenoids because of the transcriptional down-regulation of carotenogenic genes. CHYB, an enzyme that catalyses the addition of a hydroxyl residue required for esterification, was found to be a key enzyme for the accumulation of chromoplast-type carotenoids in petals.

Key words: β-ring hydroxylase, carotenoid, esterification, gene expression, *Ipomoea*, petal colour.

Introduction

Carotenoids are synthesized in chloroplasts and are essential for protecting tissues against photo-oxidative damage in the green tissues of higher plants (Britton, 1998). In flowers, carotenoids synthesized in the chromoplasts provide colour to the petals, ranging from yellow to red, in order to attract pollinators (Grotewold, 2006; Tanaka *et al.*, 2008). The colour of a flower is an important character that determines the commercial value of ornamental plants. Although abundant flower colour of Japanese morning glory (*I. nil*) can be found, flowers of
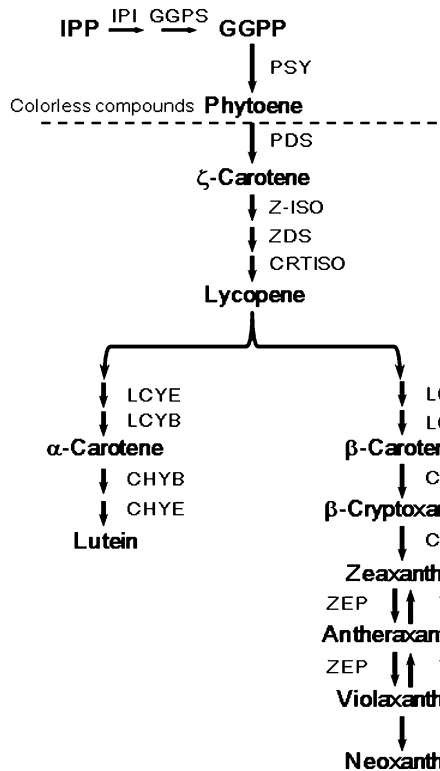
I. nil accumulate no carotenoids and lack a yellow-flowered cultivar. Despite a long history of attempts, crossbreeding aimed at producing yellow-flowered cultivars of *I. nil* has never succeeded. On the other hand, the closely related *Ipomoea* sp. and *I. obscura* have carotenoid-derived yellow and pale-yellow flowers, respectively. Hence, studying the regulatory mechanisms underlying carotenoid accumulation in *Ipomoea* plants at the molecular level will help in producing yellow-flowered cultivars by plant transformation.

Carotenoid biosynthesis starts with one isoprene unit, C_5 isopentenyl pyrophosphate (IPP; Fig. 1). Four IPPs are condensed to form C_{20} geranylgeranyl pyrophosphate (GGPP), and two GGPP molecules are converted to phytoene, the first C_{40} carotenoid, in a reaction catalysed by phytoene synthase (PSY). Phytoene is then converted via ζ-carotene to lycopene by the addition of conjugated double bonds and the conversion of *cis*- to *trans*-configurations. These reactions are catalysed by phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), carotenoid isomerase (CRTISO), and 15-*cis*-ζ-CRTISO (Z-ISO; Li *et al.*, 2007). The cyclization of the linear carotenoid lycopene catalysed by lycopene β-cyclase (LCYB) and/or lycopene ε-cyclase (LCYE) is a branch point in the pathway, leading to carotenoids with one ε- and one β-ring (α-carotene and its derivatives; ε,β-carotenoids) or two β-rings (β-carotene and its derivatives; β,β-carotenoids) (Cunningham *et al.*, 1996; Cunningham and Gantt, 2001). Subsequently, α-carotene and β-carotene are modified by hydroxylation, epoxidation, or isomerization to express a variety of structural features. Hydroxylations of the ε- and β-rings of ε,β-carotenoids are catalysed by two haem-containing cytochrome P450 mono-oxygenases, CYP97C1 (CHYE/LUT1) and CYP97A3 (CHYB/LUT5), respectively, and α-carotene is converted to lutein (Tian *et al.*, 2004; Kim and DellaPenna, 2006). Hydroxylation of the β-ring of β,β-carotenoids is catalysed by β-hydroxylase (CHYB; non-haem di-iron mono-oxygenase), and β-carotene is converted via β-cryptoxanthin to zeaxanthin (Britton, 1998; Cunningham and Gantt, 1998). Epoxidation of the β-ring of zeaxanthin, catalysed by zeaxanthin epoxidase (ZEP), yields violaxanthin. Violaxanthin is converted to neoxanthin by neoxanthin synthase (NSY). The oxygenated derivatives of carotene are called xanthophylls. In many cases, the majority of the carotenoids accumulated in flowers are xanthophylls. They are contained in chromoplasts in the esterified form (Hansmann and Sitte, 1982; Breithaupt and Bamedi, 2001).

In the past decade, nearly all of the carotenogenic genes in plants have been identified (Cunningham and Gantt, 1998; Hirschberg, 2001; Howitt and Pogson, 2006). However, the mechanisms that control carotenoid biosynthesis and accumulation in plants are largely unknown (Britton *et al.*, 2004).

Several different ways to control carotenoid accumulation in plant tissues have been reported. First, the carotenoid content depends on the plant's ability to synthesize carotenoids in the tissue. For example, white or pale-yellow cultivars or mutants of tomato (*Solanum lycopersicum*) fruits, canola (*Brassica napus*) seeds, and marigold (*Tagetes erecta*) petals show a lower expression of *PSY* than do the petals of yellow cultivars, and the transcript level is proportionate to the level of carotenoids (Fray and Grierson, 1993; Shewmaker *et al.*, 1999; Moehs *et al.*, 2001). The other mechanisms whereby carotenoid accumulation is regulated involve tissues that can synthesize carotenoids but contain trace amounts of carotenoids. One mechanism is focused on carotenoid degradation, and the other, on the sink capacity of carotenoids. In chrysanthemums (*Chrysanthemum morifolium* Ramat.), there is no significant difference between the white and yellow petals with respect to the expression levels of the carotenogenic genes (Kishimoto and Ohmiya, 2006). Synthesized carotenoids are subsequently degraded into colourless compounds by petal-specific carotenoid cleavage dioxygenase (CmCCD4a); this results in the white petal colour (Ohmiya *et al.*, 2006). The importance of sink capacity for carotenoid accumulation was first demonstrated in the *Orange* (*Or*) mutant in cauliflower (*Brassica oleracea*). Transformation of the *Or* gene into wild-type cauliflower (*or*) triggers the up-regulation of the plastid fusion and/or translocation factor (*Pftf*) and the differentiation of proplastids and other uncoloured plastids into chromoplasts; the colour of the curd tissue changes from white to orange with an increase in the levels of β-carotene (Li *et al.*, 2001; Paolillo *et al.*, 2004; Lu *et al.*, 2006).

In the present study, the patterns of carotenoid accumulation and the expression of genes related to carotenoid accumulation were compared during petal development of *Ipomoea* sp., *I. obscura*, and *I. nil* in order to clarify the factor that determines carotenoid accumulation in the petals of *Ipomoea* plants.

Fig. 1. Schematic of the carotenoid biosynthesis pathway in plants. IPP, isopentenyl pyrophosphate; IPI, IPP isomerase; GGPP, geranylgeranyl pyrophosphate; GGPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis*-ζ-CRTISO; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCYE, lycopene ε-cyclase; LCYB, lycopene β-cyclase; CHYE, ε-ring hydroxylase; CHYB, β-ring hydroxylase, ZEP, zeaxanthin epoxidase; VDE, violaxanthin deepoxidase; NSY, neoxanthin synthase.

Materials and methods

Plant materials

Yellow-flowered *Ipomoea* sp. (lineage numbers of National Bio-Resource Project [NBRP]: Q1111), pale-yellow-flowered *I. obscura* (Q1114), and white-flowered cultivars of *I. nil* (Q0260, Q0261, Q0262, Q0263, Q0686, Q1095, Q1211) were grown under a 13/11 h light/dark photoperiod in a controlled chamber at the National Institute of Floricultural Science (Tsukuba, Ibaraki, Japan). Mature leaves were used for the analysis of carotenoid composition (Fig. 2). Petal development was divided into stages 1–4 (Fig. 3A). Petals of *I. nil* were almost fully open when the lights were turned on. Stages 1, 2, and 3 refer to 96, 48, and 12 h before flower opening, respectively, and stage 4 indicates fully opened flowers. Because there was a variation in the flowering time of *Ipomoea* sp. and *I. obscura*, the developmental stage was divided according to the length of the petals. The lengths of petals of *Ipomoea* sp. and *I. obscura* were *c.* 3–5 mm at stage 1, *c.* 8–10 mm at stage 2, and *c.* 13–15 mm at stage 3. Stage 4 indicates fully opened flowers.

Carotenoid extraction and HPLC analysis

Carotenoids were extracted from leaves and petals and were analysed by HPLC, according to a method previously described by Kishimoto *et al.* (2007). The contents were calculated according to the total peak area of HPLC chromatograms at a wavelength of 450 nm and are expressed as lutein equivalents [μg g^{-1} fresh weight (FW)] of the tissue.

Isolation of total RNA and synthesis of cDNA

Total RNA was isolated from petals at each stage by using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and treated with DnaseI (Invitrogen). cDNA was synthesized from total RNA (2.5 μg) by the use of the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers.

Cloning of cDNAs encoding carotenogenic enzymes

Degenerate primers allowing amplification were designed based on sequences corresponding to highly conserved peptide regions of isopentenyl pyrophosphate isomerase (IPI), geranylgeranyl pyrophosphate synthase (GGPS), PSY, PDS, ZDS, CRTISO, LCYB, LCYE, CHYB, and carotenoid cleavage dioxygenase 1 and 4 (CCD1 and CCD4). The cDNAs encoding these proteins were

Fig. 2. Carotenoid analysis in leaves of *Ipomoea* plants. Saponified (A) and non-saponified (B) carotenoids extracted from 0.1 g fresh weight (FW) of leaves of *Ipomoea* sp. were analysed by HPLC. V, violaxanthin; N, neoxanthin; L, lutein; Z, zeaxanthin; A, antheraxanthin; β, β-carotene.

amplified by PCR using the degenerate primers. cDNAs synthesized from *Ipomoea* sp. and *I. nil* Q1211 at stage 3 were used as PCR templates. Amplified PCR products of appropriate length were cloned into the pCR2.1 vector (TA Cloning Kit, Invitrogen) and were sequenced with a Big Dye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Rapid amplification of cDNA ends (RACE) was performed to obtain the 3′ and 5′ ends of the genes from petals at stage 3 of *Ipomoea* sp. with the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Full-length cDNA sequences encoding two isoprenoid biosynthesis enzymes (*IPI*, AB499048; and *GGPS*, AB499049) and seven carotenoid biosynthesis enzymes (*PSY*, AB499050; *PDS*, AB499051; *ZDS*, AB499052; *CRTISO*, AB499053; *LCYE*, AB499054; *LCYB*, AB499055; and *CHYB*, AB499056), and partial-length cDNA sequences encoding carotenoid cleavage enzymes (*CCD1*, AB499060 and *CCD4*, AB499059) are available in the GenBank nucleotide database (see Supplementary Table S1 at *JXB* online).

Quantitative real-time PCR analysis

The transcript levels of *IPI*, *GGPS*, *PSY*, *PDS*, *ZDS*, *CRTISO*, *LCYE*, *LCYB*, *CHYB*, *CCD1*, and *CCD4* were analysed using quantitative real-time PCR (RT-qPCR) with the SYBR Premix Ex *Taq* II polymerase (TaKaRa, Ohtsu, Japan) and LightCycler System (Roche Diagnostics, Basel, Switzerland), according to the manufacturers' instructions. Each reaction (final volume, 20 μl) consisted of 10 μl 2× SYBR Premix Ex *Taq* II (TaKaRa), 0.5 μM each of the forward and reverse primers, and 2 μl of the cDNA template (corresponding to 50 ng of total RNA). The reaction mixtures were heated to 95 °C for 20 s, followed by 50 cycles at 95 °C for 5 s and 60 °C for 20 s. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. The gene-specific primers for PCR were designed using the conserved sequences among *Ipomoea* plants used in the experiments (Table 1). The *actin* primers were designed using the cDNA sequences of *I. nil actin 4* (AB054978; Yamada *et al.*, 2007). Relative standard curves describing the PCR efficiencies for each primer pair were calculated by the following equation (PCR efficiency=$10^{-1/\text{slope}-1}$), as described by Bustin *et al.* (2009). Each assay using the gene-specific primers amplified a single product of correct size with high PCR efficiency (>90%). The expression levels of *actin* and *elongation factor 1-α* were used to normalize the transcript levels of each sample. The expression patterns after normalization using *actin* or *elongation factor 1-α* as the reference gene were similar (data not shown); however, only data normalized with *actin* have been included in this paper. The plasmid solution containing each gene was serially diluted 10-fold (from 10^8 molecules μl^{-1} to 10^3 molecules μl^{-1}) and used for a standard curve assay. The transcript levels are given as the copy number per 50 ng of total RNA. The linear dynamic ranges cover at least four orders of magnitude and the level of transcripts in each reaction mixture was within the range (data not shown).

Statistically significant differences with respect to each developmental stage for values were determined by Tukey–Kramer test at the 5% level.
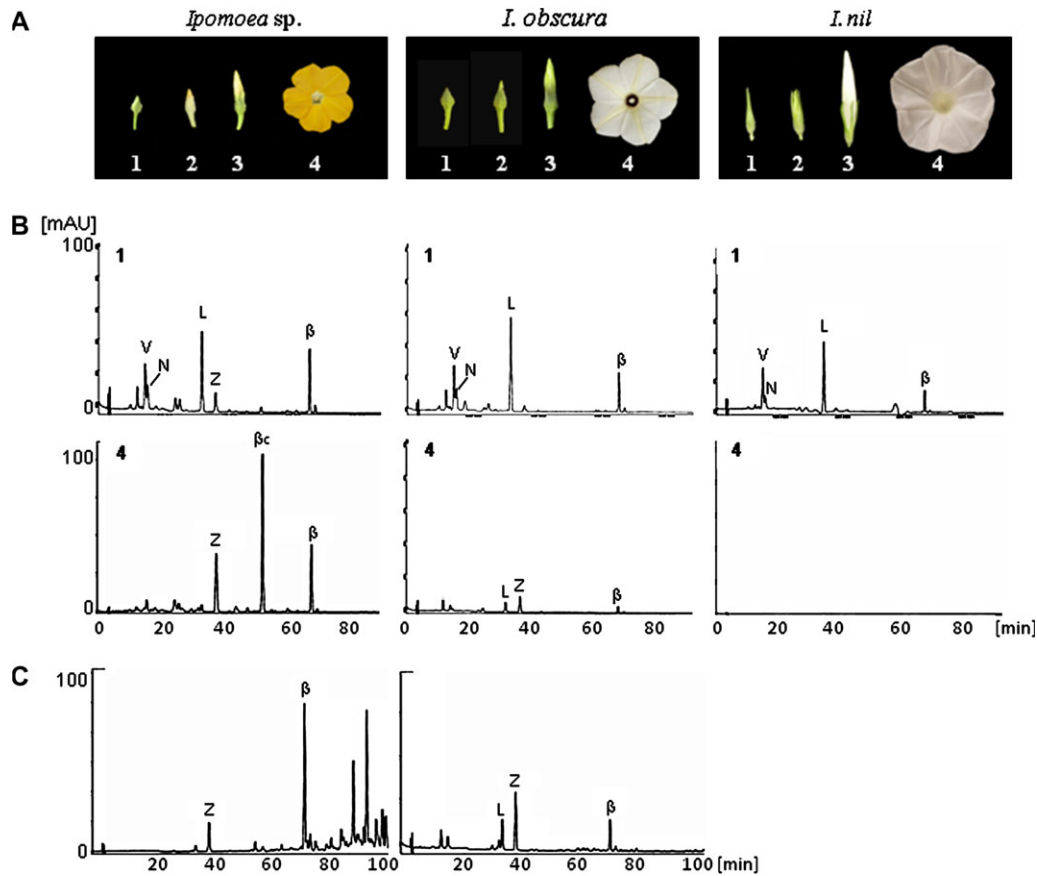
Results

Changes in carotenoid composition during petal development

HPLC chromatograms of the carotenoid extracts obtained from the leaves of *Ipomoea* sp., *I. obscura*, and *I. nil* were similar. A representative chromatogram of *Ipomoea* sp. is
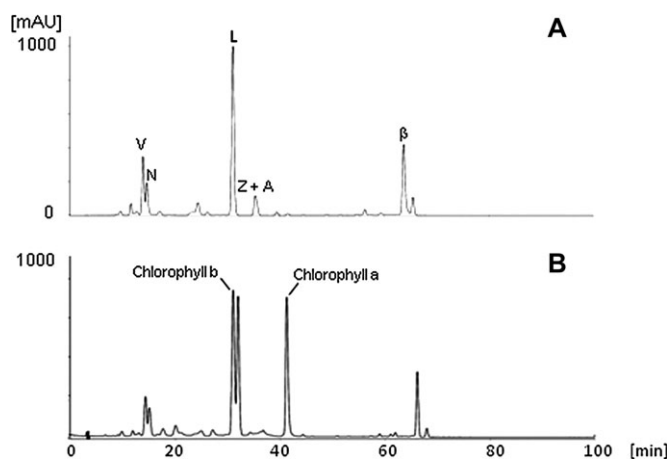
Fig. 3. Changes in carotenoid composition during petal development in *Ipomoea* plants. (A) Photographs of flowers at stages 1 and 4. HPLC elution profiles of saponified (B) and non-saponified (C) carotenoids extracted from petals of each species at various stages. V, violaxanthin; N, neoxanthin; L, lutein; Z, zeaxanthin; βc, β-cryptoxanthin; β, β-carotene. 1 and 4 indicate the sampling stages in (A).

Table 1. Primer pairs used for real-time PCR

Gene	Direction	Sequence (5′ → 3′)
IPI	Forward	TCATTGTGCGGGATGTCAGC
	Reverse	GCGGCTTCCTTGAGAGTCCC
GGPS	Forward	GGCGATTCTCACCAAGGAGC
	Reverse	CTTCCAGCGCCTTGTTCACC
PSY	Forward	GTGCAGAGTATGCAAAGACG
	Reverse	GCCTAGCCTCCCATCTATCC
PDS	Forward	CCGCCCTTTGAAGGTAGTTT
	Reverse	GTGGCCAGCATCTGCTAAAT
ZDS	Forward	TTCCTATTGGAGCACCCTTG
	Reverse	ACGAATGTCCCTCATTGCTC
CRTISO	Forward	ACCTTGCTCGTGACAGTGG
	Reverse	CAGCAACACGATGAGCACAC
LCYE	Forward	ATGGTGTGGAGGTTGAGGTG
	Reverse	ACCAAACAAGTTTCCTCAAA
LCYB	Forward	ATAGAGAGGAGGCGGCAAAG
	Reverse	GAAACAGCCGGGATGATAGA
CHYB	Forward	CCTATCGCCGACGTACCTTA
	Reverse	TCGTTTAGCCCACCAACTTC
CCD4	Forward	CGTGGGCCTTACCATCTTTT
	Reverse	AAACGTTGGGGATAACAGGAG
CCD1	Forward	GGCTCGCTTTGGAGTCCTTC
	Reverse	TCATCTCCCTCCTCCCATGC

shown in Fig. 2A. The majority of the carotenoids in leaves were lutein, violaxanthin, and β-carotene, which are essential for photosynthesis. Carotenoids in the non-saponified extract from leaves exhibited an HPLC chromatogram similar to those in the saponified leaf extract, except that chlorophyll *a* and chlorophyll *b* were detected in the non-saponified extract (Fig. 2B). The carotenoid composition in leaves was designated 'chloroplast-type carotenoid'. The total carotenoid content in the leaves of all tested cultivars was around 300 μg g^{-1} FW.

Changes in the HPLC chromatograms of carotenoid extracts during petal development in *Ipomoea* plants are shown in Fig. 3B and C and Table 2. At stage 1, all petals tested were pale green and showed the same chromatograms as chloroplast-type carotenoids, mainly showing lutein, violaxanthin, and β-carotene, albeit at lower levels than in leaves (<10 μg g^{-1} FW). In petals of *I. nil* at stage 4, the carotenoid content decreased below the detection limit, whereas small amounts of chloroplast-type carotenoids remained in *I. obscura*, and the carotenoids existed in the free form just as in leaves. In petals of *Ipomoea* sp. at stage 4, the carotenoid composition (designated 'chromoplast-type carotenoid') was completely different from the chloroplast-type carotenoids: lutein and violaxanthin

Table 2. Concentrations of carotenoid compounds in *Ipomoea* leaves and petals

Values are expressed as µg g^{-1} FW; ND, not detectable; FW, fresh weight. Each value represents the mean result from triplicate ±SD

Organ		Violaxanthin	Neoxanthin	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene
Ipomoea sp.							
Leaf		39.52±8.87	26.17±4.15	148.92±12.33	28.80±3.57	ND	89.45±10.72
Flower	Stage 1	1.96±0.48	1.14±0.30	3.73±0.66	0.98±0.54	ND	2.50±0.30
	Stage 2	5.93±1.19	10.56±1.35	11.27±0.91	36.65±7.58	17.98±7.64	15.71±5.44
	Stage 3	1.78±0.33	4.04±3.07	3.57±1.75	41.40±4.99	32.94±3.66	11.57±4.61
	Stage 4	2.84±1.59	5.19±2.27	3.61±2.26	34.14±7.16	43.25±4.60	17.71±5.10
I. obscura							
Leaf		50.73±4.28	40.63±10.96	164.81±14.40	37.16±8.33	ND	62.97±10.74
Flower	Stage 1	1.28±0.27	1.08±0.18	3.73±1.15	0.72±0.63	ND	1.43±.0.56
	Stage 2	2.66±0.45	1.23±0.49	4.41±0.63	1.81±0.53	ND	3.15±0.56
	Stage 3	1.70±0.35	1.12±0.42	2.56±1.07	1.61±1.03	ND	2.05±1.05
	Stage 4	0.52±0.34	0.46±0.26	1.00±0.13	1.11±0.13	ND	0.22±0.03
I. nil							
Leaf		34.92±9.55	19.52±1.52	129.03±19.50	19.94±10.68	ND	61.22±11.13
Flower	Stage 1	3.10±0.82	0.71±0.10	3.65±1.10	0.64±0.18	ND	1.45±0.69
	Stage 2	2.83±0.63	1.20±0.65	4.97±0.66	0.98±0.44	ND	1.03±0.06
	Stage 3	0.81±0.12	0.17±.03	0.79±0.08	0.24±0.09	ND	0.22±0.03
	Stage 4	ND	ND	ND	ND	ND	ND

levels were drastically reduced, and approximately 85% of the total carotenoids were made up of β-cryptoxanthin, zeaxanthin, and β-carotene. In addition, xanthophylls such as β-cryptoxanthin and zeaxanthin existed in the esterified form (Fig. 3C).

Figure 4 shows changes in the amounts of total carotenoids and chloroplast- and chromoplast-type carotenoids during petal development in *Ipomoea* plants. Chloroplast-type carotenoids are represented by lutein because lutein is the most abundant carotenoid in the leaves and is contained in only a trace amount in petals (Table 2). Similarly, chromoplast-type carotenoids are represented by β-cryptoxanthin. From stage 2 to stage 4, petals of *Ipomoea* sp. showed dramatic component changes in the carotenoids: chloroplast-type carotenoids were replaced by chromoplast-type carotenoids. On the other hand, petals of *I. obscura* and *I. nil* at stage 2 retained chloroplast-type carotenoids. In petals of *I. obscura* at stage 4, small amount of chloroplast-type carotenoids remained because of the slow decrease in the carotenoid level, but chromoplast-type carotenoids were not detected. In *I. nil*, the carotenoid content drastically decreased from stage 3 onwards and was entirely absent at stage 4. These results indicate that the yellow petal colour of *Ipomoea* sp. is due to newly synthesized chromoplast-type carotenoids, whereas the pale-yellow petal colour of *I. obscura* is due to the remaining chloroplast-type carotenoids synthesized at the early stage of petal development.

Analysis of carotenogenic gene expression during petal development

Genes encoding enzymes involved in isoprenoid and carotenoid biosyntheses were isolated and sequenced (see Supplementary Table S1 at *JXB* online). On the basis of the gene sequences, gene-specific primers were designed (Table 1), and

the carotenogenic gene expressions were quantified by RT-qPCR using the primers. The differences in the amounts of total RNA were normalized with respect to the levels of *actin* mRNA in each sample (Figs 5, 6).

The transcript levels of most genes in *Ipomoea* sp. and *I. obscura* were similar at stage 1 (Fig. 5). At stages 2 and 3, the transcript levels of geranylgeranyl pyrophosphate synthase (*GGPS*), *PSY*, and *PDS* drastically declined in *I. obscura* but remained high in *Ipomoea* sp. Among the carotenogenic genes tested, only *CHYB* showed a significant increase in the petals of *Ipomoea* sp. as the carotenoid composition changed from the chloroplast-type to the chromoplast-type. By contrast, the expression of *CHYB* was very low throughout petal development in *I. obscura*. In both *Ipomoea* sp. and *I. obscura*, the expression levels of all genes were lower at stage 4 than those during the early stage of petal development.

All seven white-flowered cultivars tested of *I. nil* showed similar expression patterns. The results of two representative cultivars (Q0686, and Q1211) are shown in Fig. 5. All carotenogenic genes, except *CHYB*, were expressed at stages 1 and 2 in petals of *I. nil*, albeit at low levels. At stages 3 and 4, the expressions of most genes were down-regulated. In particular, the *CHYB* transcript level was extremely low in *I. nil* at all stages.

Analysis of the expression levels of genes involved in caroteniod cleavage

To examine whether carotenoid degradation is involved in the formation of the white colour in *Ipomoea* petals, the transcript level of genes encoding carotenoid cleavage dioxygenases (CCDs) were analysed by RT-qPCR (Fig. 6). Among the four types of *CCD* homologues, *CCD1*, *CCD4*, *CCD7*, and *CCD8*, reported in *Arabidopsis* (Tan *et al.*,
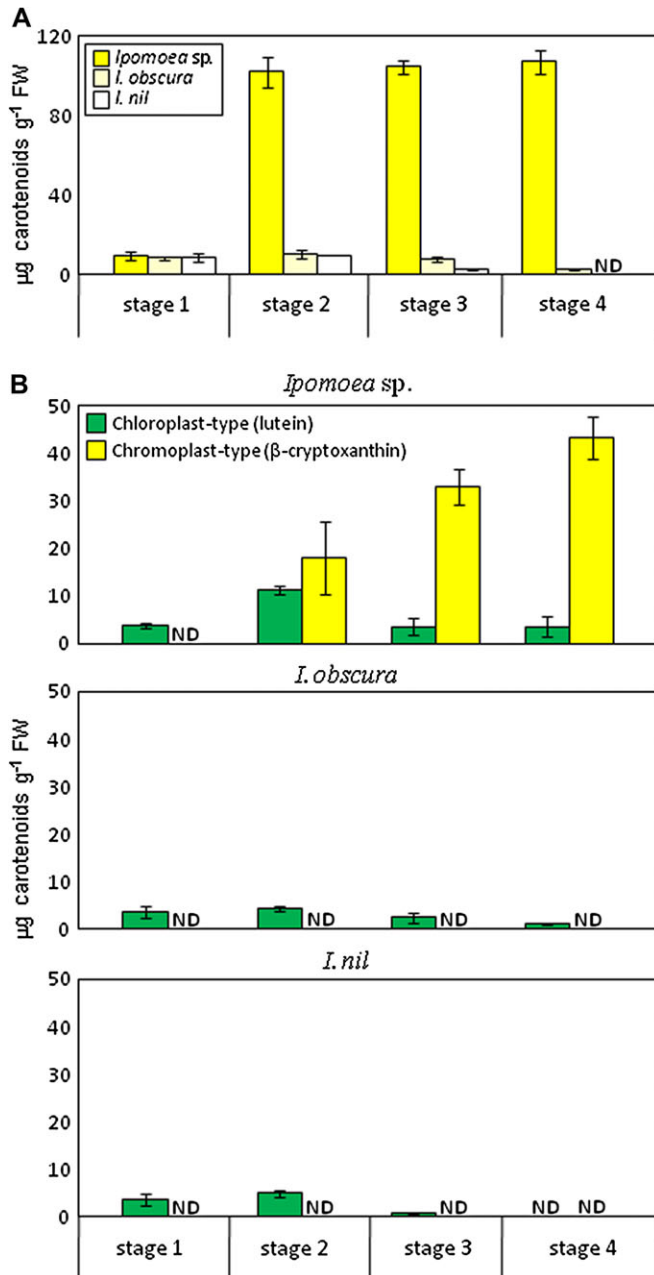
Fig. 4. Changes in carotenoid composition during petal development. Total carotenoid concentration (A) and chloroplast- and chromoplast-type carotenoid content (B) in petals of *Ipomoea* sp., *I. obscura*, and *I. nil* at stages 1 to 4. Chloroplast- and chromoplast-type carotenoids are represented by lutein and β-cryptoxanthin, respectively. Each bar represents the mean result from triplicate ±SD. ND, not detectable.

2003), it was possible to obtain cDNAs encoding CCD1 and CCD4 homologues from *Ipomoea* plants.

All the *Ipomoea* plants tested showed a low level of *CCD1* expression during the course of petal development. The expression pattern of *CCD4* during petal development differed among the *Ipomoea* plants. *CCD4* was expressed at a relatively high level in petals of *Ipomoea* sp. throughout development. In *I. obscura*, wherein petals at stage 4 contained chloroplast-type carotenoids, *CCD4* expression

declined remarkably during petal development. By contrast, *CCD4* expression remained high at stages 3 and 4 in *I. nil*. These results suggest that the pale-yellow petal colour of *I. obscura* derived from chloroplast-type carotenoids is caused by a reduced level of *CCD4* expression.

Discussion

Changes in carotenoid composition during petal development

The leaves of most plants show similar carotenoid profiles, containing both ε,β-carotenoids and β,β-carotenoids (Goodwin and Britton, 1988). Carotenoids essential for photosynthesis, such as lutein, violaxanthin, and β-carotene, are generally found in leaves. Because they are contained in chloroplasts, these were designated as 'chloroplast-type carotenoids.' By contrast, the carotenoid composition in flowers varies depending on plant species, and carotenoids accumulating in chromoplasts are in a more oxidized form than chloroplast-type carotenoids (Deli *et al.*, 1988; Goodwin and Britton, 1988; Kull and Pfander, 1997; Maoka *et al.*, 2000; Tai and Chen, 2000; Kishimoto *et al.*, 2005). The present results show that in all plants tested, petals contained chloroplast-type carotenoids at the early developmental stage (Fig. 3). At the late stage of petal development, the levels of chloroplast-type carotenoids decreased, and high levels of carotenoids, of which the composition differed from that of chloroplast-type carotenoids, accumulated only in *Ipomoea* sp.: the accumulated carotenoids included β,β-carotenoids such as β-cryptoxanthin, zeaxanthin, and β-carotene (designated as 'chromoplast-type carotenoids'). In addition, the chromoplast-type carotenoids were mostly present in the esterified form, while the chloroplast-type carotenoids existed in the free form. The importance of esterification for carotenoid sequestration into chromoplasts has been well documented in pepper fruits (Minguez-Mosquera and Hornero-Mendez, 1994*a*, *b*; Hornero-Mendez and Minguez-Mosquera, 2000). Xanthophyll esterification was accompanied by the transition of chloroplasts (green fruits) into chromoplasts (red fruits). Previous studies have shown that, in flowers of marigold and *Eustoma*, a significant proportion of carotenoids are esterified during petal development (Moehs *et al.*, 2001; Nakayama *et al.*, 2006). Hence, it is assumed that esterification is an important process for the sequestration of carotenoids into chromoplasts in *Ipomoea* sp.

Differences in carotenogenic gene expression during petal development

When the carotenogenic gene expression in petals of *Ipomoea* plants was analysed in relation to compositional changes in carotenoids occurring during petal development, the most prominent feature was the difference in *CHYB* expression among *Ipomoea* sp., *I. obscura*, and *I. nil* (Fig. 5). *CHYB* expression drastically increased at stages 2 and 3 in petals of *Ipomoea* sp., whereas it remained extremely low
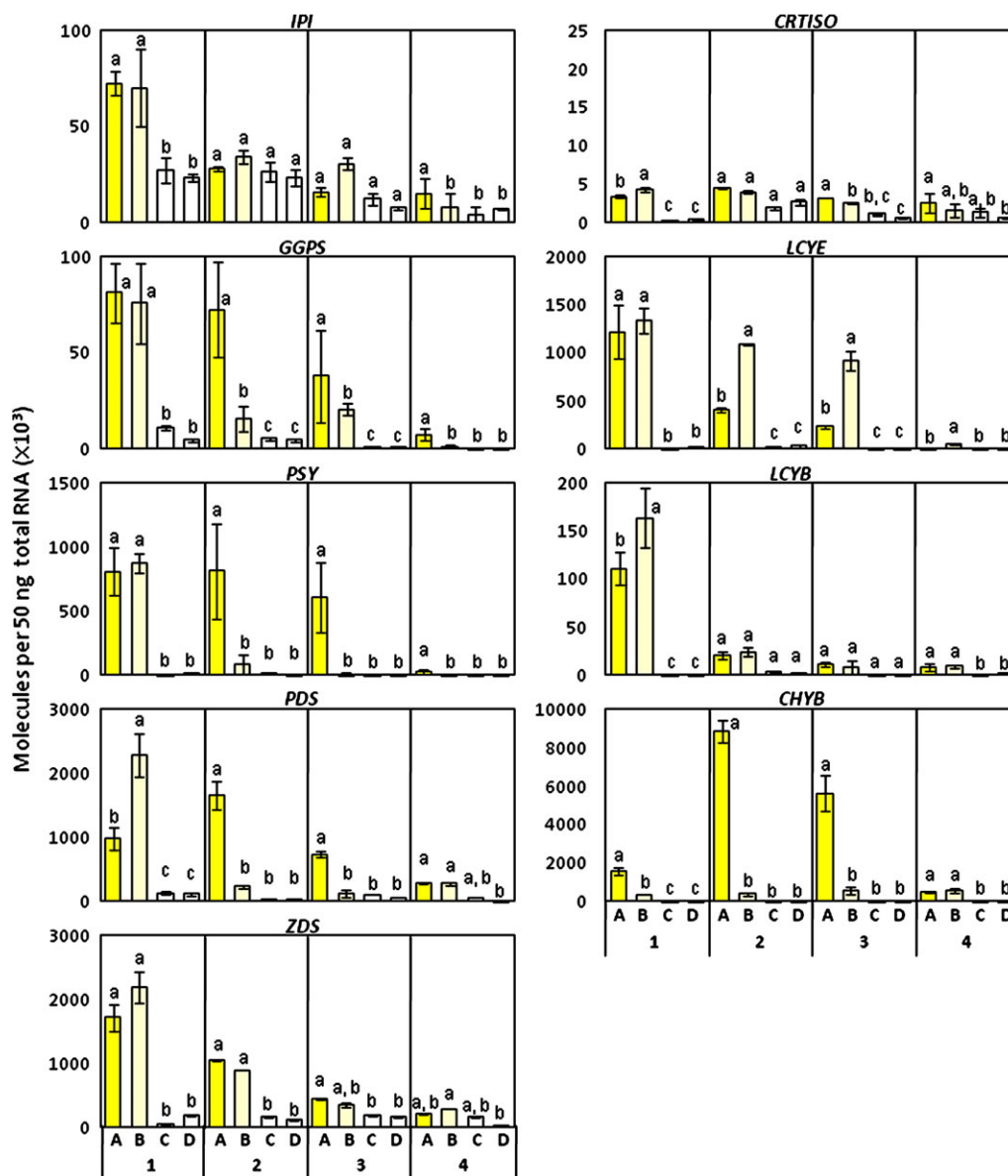
Fig. 5. Changes in carotenogenic gene expression during petal development. RT-qPCR analyses were performed in triplicate by using gene-specific primers, and the expression levels were normalized against mRNA levels of *actin*; mean values (SE) are shown. 1, 2, 3, and 4 indicate sampling stages. (A) *Ipomoea* sp. (Q1111); (B) *I. obscura* (Q1114); (C) *I. nil* (Q0686); (D) *I. nil* (Q1211). *IPI*, IPP isomerase; *CRTISO*, carotenoid isomerase; *GGPS*, geranylgeranyl pyrophosphate synthase; *LCYE*, lycopene ε-cyclase; *PSY*, phytoene synthase; *LCYB*, lycopene β-cyclase; *PDS*, phytoene desaturase; *CHYB*, β-ring hydroxylase; *ZDS*, ζ-carotene desaturase. Different letters within each stage indicate significant differences by Tukey-Kramer test (*P* <0.05).

throughout the course of petal development both in *I. obscura* and *I. nil*. There is increasing evidence that CHYB plays an important role in carotenoid accumulation in the chromoplast of various plants. The *wf* mutant in tomato, which produces the white flower phenotype is caused by a mutation in *CrtR-b2*, a *CHYB* homologue that is specifically expressed in petals (Galpaz *et al.*, 2006). The *wf* mutant shows a significant decrease in the levels of β-carotene derivatives in petals, whereas the transcript levels of genes encoding the rate-limiting enzymes involved in isoprenoid and carotenoid biosyntheses, such as 1-deoxy-D-xylulose-5-phosphate synthase (DXS), PSY, GGPS, PDS, and ZDS show the same transcript levels as in the petals of wild-type plants. In tepals of Asiatic hybrid lily (*Lilium* spp.), the expression level of the *CHYB* homologue in various cultivars correlated with the carotenoid content and more closely than any other carotenogenic genes (Yamagishi *et al.*, 2009). In *Arabidopsis*, the homologue of *CHYB* was not expressed in the floral organ (Kim *et al.*, 2009). Hydroxylation is an important process for the esterification of carotenoids, which in turn is necessary for carotenoid sequestration and stabilization in the chromoplast. The up-regulation of *CHYB*, which encodes for an enzyme that catalyses the addition of hydroxyl residues required for esterification, may be a key event in carotenoid accumulation in the chromoplast.
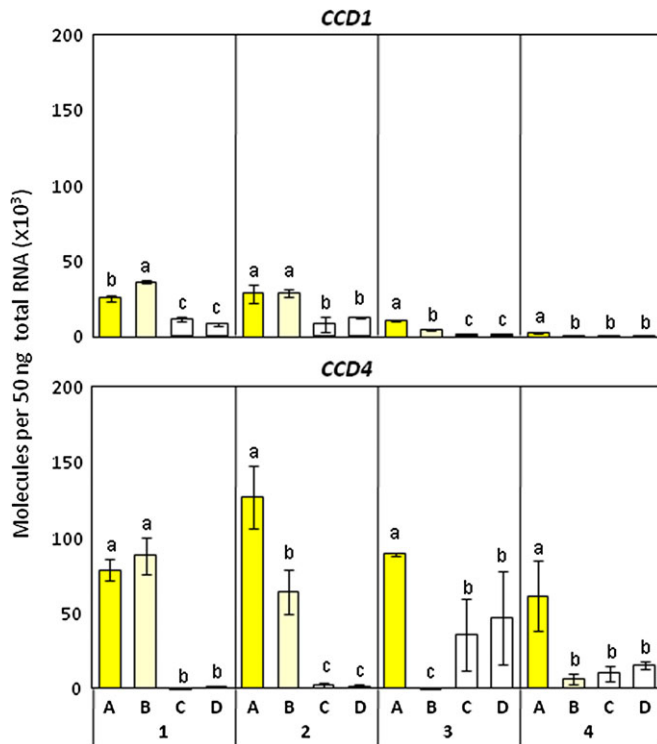
Fig. 6. Changes in the expression pattern of *CCD1* and *CCD4* during petal development. RT-qPCR analyses were performed in triplicate by using gene-specific primers, and the expression levels were normalized against mRNA levels of *actin*; mean values (SE) are shown. Sampling stages and plant materials are the same as described in Fig. 5. Different letters within each stage indicate significant differences by Tukey–Kramer test (P <0.05).

Generally, it is thought that the apportionment of substrates into the pathways leading to the synthesis of α- and β-carotenoids is determined simply by the relative amounts or activities of LCYB and LCYE. For example, in chrysanthemums, the difference in carotenoid components between petals and leaves is caused by the different expression levels of *LCYB* and *LCYE* (Kishimoto and Ohmiya, 2006). In addition, the repression of *LCYE* expression increases the β,β-carotenoid content in potato tubers and canola seeds (Diretto *et al.*, 2006; Yu *et al.*, 2008). In *Ipomoea* sp., the decrease in the levels of chloroplast-type carotenoids is accompanied by a decrease in the level of *LCYE* transcripts. The level of *LCYB* transcripts, however, showed no correlation with the accumulation of chromoplast-type carotenoids (mainly β,β-carotenoids) and did not considerably differ from the *LCYB* transcript level in *I. obscura*. These results suggest that CHYB and not LCYB functions as a key enzyme in the synthesis of chromoplast-type carotenoids in the petals of *Ipomoea* sp.

Expression patterns of genes involved in carotenoid degradation during petal development

In chrysanthemums, CmCCD4a contributes to the white colour of petals by cleaving carotenoids into colourless

compounds (Ohmiya *et al.*, 2006). However, little data on whether the same mechanism is applicable to other plant species are available. In petals of *Ipomoea* sp., the *CCD4* expression was relatively high at stages 2 and 3 when the level of chromoplast-type carotenoids was increased. In addition, both *Ipomoea* sp. and *I. nil* showed high levels of expression of *CCD4* during the degradation of chloroplast-type carotenoids. In petals of *I. obscura*, which accumulate chloroplast-type carotenoids even at stage 4, *CCD4* drastically decreased as the petals matured. These results suggest that in *Ipomoea* plants, *CCD4* is not involved in the degradation of chromoplast-type carotenoids but is involved in the degradation of chloroplast-type carotenoids.

To examine whether the carotenoid degradation mechanism is responsible for white colour formation in petals of *Ipomoea* plants, *Ipomoea* sp. and *I. obscura* were crossed (see Supplementary Fig. S1 at *JXB* online). It is thought that the white petal colour would be dominant over the yellow colour if carotenoid degradation is responsible for the white colour formation of petals in *Ipomoea*. However, the petals of all progenies obtained by the crossing were yellow and accumulated chromoplast-type carotenoids, suggesting that carotenoid cleavage is not responsible for the lack of chromoplast-type carotenoids in *I. obscura*.

The sink capacity of carotenoids is another factor to be considered. The importance of sink capacity for carotenoid accumulation was first demonstrated in cauliflower *Or* mutants (Lu *et al.*, 2006). Transformation of the *Or* gene into wild-type cauliflower (*or*) exhibit increased expression of *Pftf*, but not of carotenoid biosynthesis genes (Li *et al.*, 2001; Lu *et al.*, 2006). The colour of the curd tissue of transformants changes from white to orange with an increase in the levels of β-carotene. In *Ipomoea* plants, however, there was no significant correlation between the level of *Pftf* transcripts and the carotenoid content in petals (data not shown).

These results suggest that the carotenoid content in petals of *Ipomoea* plants is related neither to carotenoid degradation activity nor to the sink capacity of carotenoids, but instead to the transcriptional down-regulation of carotenogenic genes, especially *CHYB*.

Putative mechanism for the regulation of carotenogenic gene expression in lpomoea petals

In the present study, it is shown that petals of *I. nil* and *I. obscura* accumulated little, if any, chromoplast-type carotenoids because of the transcriptional down-regulation of carotenogenic genes, in particular, the transcriptional down-regulation of *CHYB*. Two hypotheses were postulated to explain such down-regulation. (i) A chromoplast-specific carotenoid biosynthesis pathway exists in *Ipomoea* sp. but not in *I. nil* or *I. obscura*. (ii) Petal-specific down-regulation of the carotenoid biosynthesis pathway in *I. nil* and *I. obscura* causes low carotenoid production.

Reports of genes encoding chromoplast-specific carotenoid biosynthesis enzymes suggest a chromoplast-specific carotenoid biosynthesis pathway (Bartley *et al.*, 1992; Fray

and Grierson, 1993; Pecker *et al.*, 1996; Ronen *et al.*, 2000). Two types of *CtrR-b* genes, which encode non-haem β-carotene hydroxylase, exist in tomato and pepper, one of which is specifically expressed in the fruit (Bouvier *et al.*, 1998; Galpaz *et al.*, 2006). However, in this study, only one type of *CHYB* gene was isolated from *Ipomoea* sp. The predicted polypeptide sequence of CHYB in *Ipomoea* sp. shares 87% similarity with both tomato CRTR-B1 (chloroplast-specific CHYB) and CRTR-B2 (chromoplast-specific CHYB) (see Supplementary Fig. S2 at *JXB* online). Because the chloroplast transit peptide (cTP) sequences of CRTR-B1 and CRTR-B2 shared only 56% similarity, the cTP sequence of CHYB in *Ipomoea* sp. was compared with that of tomato CRTR-B1 and CRTR-B2. The cTP of *Ipomoea* CHYB shared 56% and 59% identity with the cTP sequences of tomato CRTR-B1 and CRTR-B2, respectively (see Supplementary Fig. S2 at *JXB* online). CHYB cannot be categorized as a CRTR-B1 type or a CRTR-B2 type on the basis of its sequence; hence, it is assumed that CHYB has a redundant function in *Ipomoea* plants. The existence of chromoplast-specific PSY and LCYB has also been reported previously (Fray and Grierson, 1993; Pecker *et al.*, 1996). However, only one type each of PSY and LCYB was isolated from *Ipomoea* sp. Moreover, the EST database of *I. nil* holds only one contig for *CHYB*, *PSY*, and *LCYB* (http://ipomoeanil.nibb.ac.jp/). Although the possibility cannot be excluded that genes encoding chromoplast-specific enzymes exist in *Ipomoea* plants, the formation of white petal colour in *Ipomoea* plants may be controlled by a mechanism different from that in the tomato *wf* mutant.

It is well known that the carotenoid content in plant tissues, especially flowers and fruits, varies widely within the same species. In marigold, differences in petal colour, from pale-yellow to dark orange, are caused by different levels of lutein accumulation. In a previous study, it was found that the lutein content correlates well with the level of *PSY* and *DXS* transcripts (Moehs *et al.*, 2001). It has also been demonstrated that PSY in tomato fruits (Pecker *et al.*, 1992; Giuliano *et al.*, 1993; Fraser *et al.*, 1994) and GGPS, PSY, and PDS in pepper (Hugueney *et al.*, 1996) are rate-limiting enzymes in carotenoid biosynthesis. The *CHYB* transcript level in petals of *Ipomoea* sp. drastically increased with an increase in chromoplast-type carotenoid accumulation at stages 2 and 3, but that of *I. obscura* and *I. nil* was significantly low throughout petal development. Moreover, at stages 2 and 3, the transcript levels of *GGPS*, *PSY*, and *PDS* remarkably decreased in petals of *I. obscura* and *I. nil* but remained high in those of *Ipomoea* sp. (Fig. 5). These results suggest that CHYB is mainly responsible for the regulation of chromoplast-specific carotenoid accumulation in petals of *Ipomoea* plants, and GGPS, PSY, and PDS are also involved the regulation process (Fig. 7).

Recent studies have shown that the transcriptional regulation of carotenoid biosynthesis enzymes is the major factor controlling carotenogenesis in plant tissues (Fraser and Bramley, 2004; Sandmann *et al.*, 2006). One possible mechanism for the repression of multiple carotenogenic genes in petals of *I. nil* is feedback inhibition at the
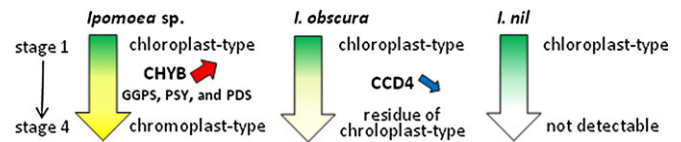
Fig. 7. Schematic representation of the mode of carotenoid accumulation in petals of *Ipomoea* plants. Differences in carotenoid accumulation patterns and related enzymes during petal development among *Ipomoea* plants are shown. CHYB, β-ring hydroxylase; GGPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; CCD, carotenoid cleavage dioxygenase.

transcriptional level caused by repression of *CHYB* expression. In the *wf* mutant of tomato, the expression level of genes upstream of the carotenoid biosynthesis pathway, such as of *DXS*, *GGPS*, *PSY*, and *PDS*, is not affected by *CHYB* repression (Galpaz *et al.*, 2006). Little data are available regarding feedback inhibition of the carotenoid pathway by an enzyme downstream of the pathway. Transgenic plants overexpressing *CHYB* will provide an answer to whether *CHYB* influences the expression level of upstream genes.

Another possible mechanism is down-regulation of multiple carotenogenic genes caused by a common transcription factor. In *Arabidopsis*, only one *PSY* gene exists. AtRAP2.2 was isolated as a transcription factor that binds to the *cis*-acting element ATCTA in the promoter of *PSY*. The ATCTA element is also present in the promoter of *PDS*, which suggests that a common regulatory mechanism exists in both genes (Welsch *et al.*, 2007). Co-repression of multiple carotenogenic genes during petal development in *I. obscura* and *I. nil* suggests the existence of a transcription factor common to multiple carotenogenic genes; however, there are no reports on the mechanism regulating the entire carotenoid biosynthesis pathway. Understanding the mechanism underlying the regulation of carotenoid biosynthesis in plant tissues will accelerate the process of engineering this pathway, which has only partly been achieved thus far.

Supplementary data

Supplementary data are available at *JXB* online.
Supplementary Table S1. Genes encoding isoprenoid and carotenoid biosynthesis enzymes isolated from *Ipomoea* sp.
Supplementary Fig. S1. Crossing between *Ipomoea* sp. and *I. obscura*.
Supplementary Fig. S2. Multiple sequence comparison of CHYB among various plant species.

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