

The carotenoid cleavage dioxygenase 4 (*CmCCD4a*) gene family encodes a key regulator of petal color mutation in chrysanthemum

Satoshi Yoshioka · Ryutaro Aida ·
Chihiro Yamamizo · Michio Shibata ·
Akemi Ohmiya

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Abstract It has long been proposed that white-flowered chrysanthemums (*Chrysanthemum morifolium* Ramat.) have a single dominant gene that inhibits carotenoid formation or accumulation in ray petals. However, the precise function of the proposed gene was unknown. We previously isolated a gene encoding carotenoid cleavage dioxygenase 4, designated *CmCCD4a*, which is specifically expressed in the ray petals of white-flowered chrysanthemums. Because *CmCCD4a* was a strong candidate for the single dominant gene, we analyzed the relationship between *CmCCD4a* expression and carotenoid content in two sets of petal color mutants. Here, we show that *CmCCD4a* represents a small gene family containing

at least four members. Two of them, *CmCCD4a-1* and *CmCCD4a-2*, were highly expressed in ray petals of two taxa with low carotenoid levels. In petal color mutants derived from these taxa, increases in carotenoid levels accompanied decreases in *CmCCD4a* expression levels in ray petals. Two different circumstances reduced the levels of *CmCCD4a* expression in the mutants: either a *CmCCD4a* gene was lost from the genome or the expression of a *CmCCD4a* gene was suppressed. In the latter case, suppression may be caused by the loss of a function that normally enhances *CmCCD4a* transcription. A stepwise decrease in the amount of *CmCCD4a* expression in either L1 or L2 resulted in a corresponding stepwise increase in the carotenoid content in ray petals. From these results, we propose that *CmCCD4a* expression is the key factor that controls the carotenoid content in ray petals of chrysanthemum.

Accession numbers The genomic sequences are available in the GenBank nucleotide database under the accession numbers AB627797 (*CmCCD4a-1*), AB627798 (*CmCCD4a-2*), AB627800 (*CmCCD4a-3*), and AB627802 (*CmCCD4a-4*). The cDNA sequences are available under the accession numbers AB247158 (*CmCCD4a-1*), AB627799 (*CmCCD4a-2*), AB627801 (*CmCCD4a-3*), and AB627803 (*CmCCD4a-4*).

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S. Yoshioka · R. Aida · C. Yamamizo ·
M. Shibata · A. Ohmiya (✉)
National Agriculture and Food Research Organization
(NARO), Institute of Floricultural Science, Tsukuba,
Ibaraki 305-8519, Japan
e-mail: ohmiya@affrc.go.jp

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Introduction

The major pigments in the ray petals of chrysanthemum (*Chrysanthemum morifolium* Ramat.) are lutein derivatives (yellow carotenoids) and cyanidin derivatives (red anthocyanins) (Nakayama et al. 1997; Kishimoto et al. 2004). Combinations of these pigments produce a wide range of ray petal colors,

including red, orange, yellow, pink, and white (Kawase and Tsukamoto 1976; Kishimoto et al. 2007).

Petal color mutants of outstanding chrysanthemum cultivars have sometimes been developed as new cultivars (Wasscher 1956). Petal color mutations from white to yellow occasionally occur, but mutations in the opposite direction (yellow to white) are rare (Machin and Scope 1978). It has been postulated that a single dominant gene that inhibits carotenoid formation or accumulation acts in the ray petals of white-flowered chrysanthemum (Stewart and Derman 1970; Jordan and Reinmann-Philipp 1983; Hattori 1991; Boase et al. 1997). We isolated a gene encoding carotenoid cleavage dioxygenase 4, designated *CmCCD4a*, that is specifically expressed in ray petals of white-flowered chrysanthemum (Ohmiya et al. 2006). Suppression of *CmCCD4a* expression by RNA interference (RNAi) in white-flowered chrysanthemum produced yellow-flowered transformants (Ohmiya et al. 2006, 2009). In addition, all of the F₁ progeny obtained by crossing between white-flowered wild chrysanthemums containing *CmCCD4a* orthologs and a yellow-flowered cultivar without *CmCCD4a* had white ray petals, indicating that *CmCCD4a* is a dominant gene that was homozygous in the white-flowered wild chrysanthemums (Yoshioka et al. 2010). These results made *CmCCD4a* a strong candidate for the single dominant gene.

CmCCD4a may represent a small gene family rather than a single gene, because chrysanthemum cultivars are hexaploid ($2n = 6x = 54$; Endo 1969; Shibata et al. 1998). On the other hand, it has been proposed that carotenoid accumulation in ray petals is controlled by a single factor (Stewart and Derman 1970; Jordan and Reinmann-Philipp 1983; Hattori 1991; Boase et al. 1997). The fact that petal color mutation from white to yellow sometimes occurs in some white-flowered cultivars, such as ‘Marble’, ‘Daisy’, and ‘Indianapolis’ (Yoder Bros Inc. 1967), suggests that few copies of *CmCCD4a* function in such cultivars. On the other hand, there are white-flowered cultivars such as ‘Jimba’ and ‘Sei-Marine’ in which petal color mutations rarely occur, suggesting that multiple copies of *CmCCD4a* function in such cultivars. In addition, mutations leading to yellow flower color generally progress in a stepwise pattern from white to pale yellow to deep yellow. The copy number of functional *CmCCD4a* genes might explain this stepwise increase in carotenoid

levels; nevertheless, little data on *CmCCD4a* has been available.

In this study, we sought to clarify the involvement of *CmCCD4a* in regulating carotenoid accumulation in ray petals of chrysanthemum. Two types of petal color mutants leading to increases in carotenoid content were used for the analysis: one was obtained by somatic variation during tissue culture, the other by bud-sport mutation. We analyzed the relationship between *CmCCD4a* transcript level and carotenoid content in ray petals of these mutants. From the results, we propose a mechanism that could cause an increase in carotenoid content in chrysanthemum ray petals during the process of petal color mutation.

Materials and methods

Plant materials

The chrysanthemum cultivars used in this study were obtained from the genetic resource stocks of the National Institute of Floricultural Science (Tsukuba, Ibaraki, Japan). The ‘Marble’ cultivars are a series of bud sports with various petal colors; the ones used in this study were ‘Pink Marble (PM)’, ‘Apricot Marble (AM)’, ‘Bronze Marble (BrM)’, ‘Blue Marble (BM)’, ‘Coral Marble (CM)’, ‘Orange Marble (OM)’, ‘White Marble (WM)’, ‘Polished Marble (PoM)’, and ‘Florida Marble (FM)’. ‘Paragon’ is a white-flowered cultivar. Six independent petal color mutants (*cam1–cam6*) were found among regenerants derived from ‘94-765’, a tissue culture line obtained from Seikoen Co., Ltd., Japan (Fig. 1).

RT-PCR-mediated cloning of *CmCCD4a* homologs

Total RNA was isolated from ray petals of fully opened flowers by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and then treated with DNase I (Invitrogen) according to the manufacturer’s instructions. cDNAs were synthesized from the total RNA by using the Superscript First-Strand Synthesis System (Invitrogen). For isolation of homologs of *CmCCD4a*, RT-PCR was performed with ray petal cDNA of each petal color mutant as the template and the primer set CCD4a-F and CCD4a-R (Table 1) using Advantage 2 DNA Polymerase (Clontech, Mountain View, CA,

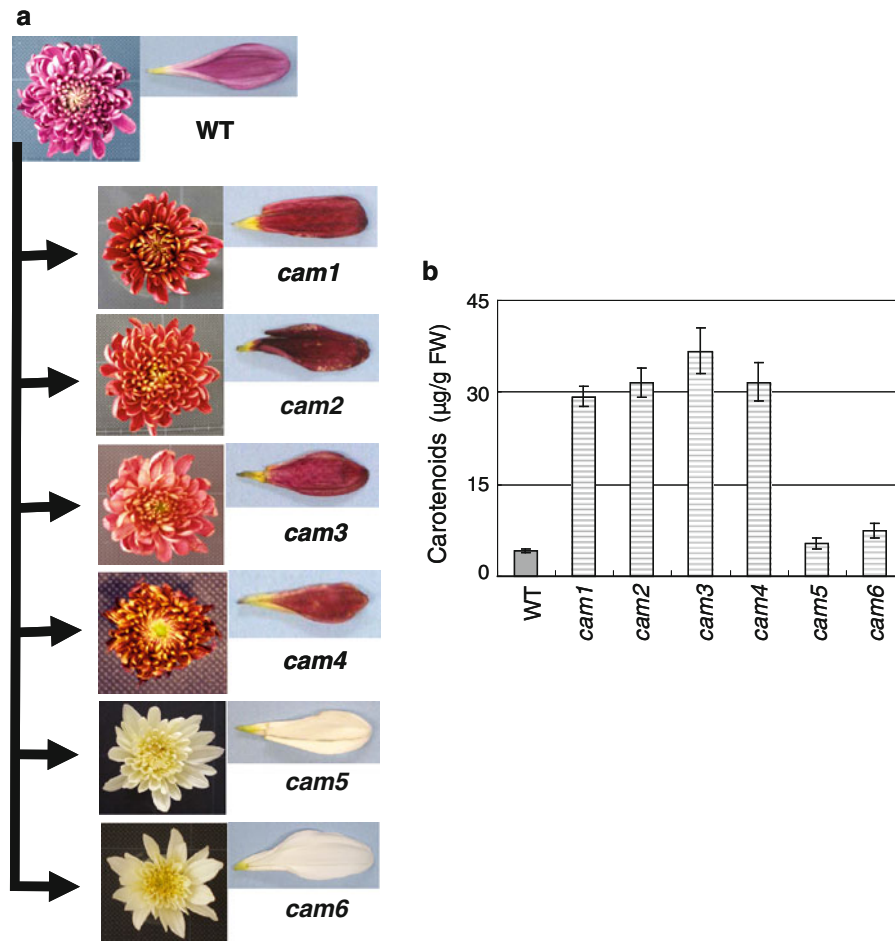


Fig. 1 Ray petal colors and carotenoid contents in wild type of chrysanthemum laboratory line ‘94-765’ (WT) and its petal color mutants, *cam1*–*cam6*. **a** Whole flowers and adaxial sides of ray petals. **b** Carotenoid contents in ray petals. Error bar \pm SEM ($n = 3$)

USA). The PCR conditions were as follows: 95°C for 2 min; and 35 cycles at 95°C for 10 s, 64°C for 20 s, and 72°C for 90 s. PCR fragments were cloned into the pCR2.1 vector (Original TA Cloning Kit, Invitrogen) and sequenced with a Big Dye Terminator v. 3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Cloning of genomic *CmCCD4a* homologs by PCR

Genomic DNA was isolated from mature leaves by the cetyl trimethyl ammonium bromide method (Murray and Thompson 1980). The promoter region of *CmCCD4a-1* was amplified from chrysanthemum genomic DNA by thermal asymmetric interlaced PCR (Liu et al. 1995). Three nested primers complementary to the coding sequence of *CmCCD4a-1*

(4a-1-N1, 4a-1-N2, 4a-1-N3) and an arbitrary degenerate primer (AD) were used (Liu et al. 1995). The genomic sequences including promoter and coding regions of *CmCCD4a-2*, -3, and -4 were isolated using primers complementary to the sequence of *CmCCD4a-1*. The sequences of primers used for the genomic PCR are listed in Table 1. The PCR conditions were 95°C for 2 min; and 35 cycles at 95°C for 10 s, 64°C for 20 s, and 68°C for 90 s. A predicted plastid-targeting peptide was identified by the iPSORT program (<http://hc.ims.u-tokyo.ac.jp/iPSORT/>).

Gene expression analyses

Levels of *CmCCD4a* transcripts were analyzed by reverse-transcription quantitative real-time PCR (RT-qPCR) using SYBR Premix Ex *Taq* II (TaKaRa,

Table 1 Primers used in this study

CmCCD4a primers for RT-PCR-mediated cloning
 CCD4a-F: 5'-CAGACAGAGAAAGATGCGGAATCA-3'
 CCD4a-R: 5'-CTCATAAATGATGTGTCTAGTAGGAG-3'

Primers for thermal asymmetric interlaced (TAIL)-PCR
 4a-1-N1: 5'-CGGTGTTTACGAGTTGAAGATA-3'
 4a-1-N2: 5'-AAACCCTAAGTGGTGGATCAAT-3'
 4a-1-N3: 5'-CCCTCAATCATTTCACATTAG-3'
 AD: 5'-NGTCGASWGANAWGAA-3'

CmCCD4a-1- and *CmCCD4a-2*-specific primers
 for genomic PCR
 Pro4a-1-F: 5'-GGGTTCTCGCACCTAAGGAATA-3'
 Pro4a-1-R: 5'-GTATGGAAATAAGCTACATACCCA-3'
 Pro4a-2-F: 5'-GAGTAAAATTGGGTTCTCTAGC-3'
 Pro4a-2-R: 5'-GCTACATAGCAAATTTACATAACAA-3'

CmCCD4a-1- and *CmCCD4a-2*-specific primers
 for RT-qPCR
 4a-1-F: 5'-ACGACGACCACTAGCACCGC-3'
 4a-1-R: 5'-ACAGGAGAAAAGCTATGTGATAAAA-3'
 4a-2-F: 5'-AGGAAGATCAGCCCTTACCAAC-3'
 4a-2-R: 5'-AAAATGTGGCTCTTCTTTTGA-3'

Chrysanthemum *actin* primers for RT-qPCR
 Actin-F: 5'-CTTGCCTTTGGATCTTGCTGGTCGTGA-3'
 Actin-R: 5'-AGCAGCTTCCATCCAATCATAGACGG-3'

Ohtsu, Japan). Reactions were carried out with a LightCycler system (Roche Diagnostics, Basel, Switzerland). The RT-qPCR conditions were as follows: 95°C for 20 s; and 45 cycles at 95°C for 4 s, 64°C for 8 s, and 68°C for 20 s. Known concentrations of plasmid DNAs were used for drawing standard curves. The expression level of *actin* (GenBank accession no. AB205087) was used to normalize the transcript level in each sample. The sequences of primers used for RT-qPCR are listed in Table 1.

Carotenoid extraction, quantification, and HPLC analysis

Ray petals (0.5 g) were ground in acetone. Before grinding, the basal part of each (up to ~5 mm from the bottom) was removed, because the color was different from the rest of the petal (Fig. 1a). The extracts were partitioned between diethyl ether and aqueous NaCl. The absorbance of the diethyl ether layer at a wavelength of 450 nm was measured. Carotenoid content was calculated by using the $E_{1\%}^{1\text{cm}}$ value of lutein

(Britton 1995) and expressed as micrograms of lutein equivalent per gram fresh weight ($\mu\text{g/g}$ FW) of tissue. Carotenoids were extracted from petals and were analyzed by high-performance liquid chromatography (HPLC; X-LC, JASCO, Tokyo, Japan) with a YMC carotenoid column ($4.6 \times 250 \text{ mm}^2$; YMC, Kyoto, Japan), according to Kishimoto et al. (2007). Peaks were identified by comparing retention times and absorbance spectra with those of previously identified compounds in ray petals of chrysanthemum (Kishimoto et al. 2004; Kishimoto and Ohmiya 2006) and with those of standard compounds.

Microscopic observation

Fresh ray petals were sliced longitudinally with paired double-edge razor blades. The sections were examined through a Provis-AX70 light microscope (Olympus, Tokyo, Japan). Photographs were taken with DP Controller software (Olympus).

Results

Cloning of *CmCCD4a* homologs

To isolate genes encoding *CmCCD4a*, we performed RT-PCR using cDNA of ray petals of 'Paragon', a white-flowered chrysanthemum cultivar, as a template. At least 4 types of *CmCCD4a* homologs with sequences >90% identical to one other were isolated: these genes were designated *CmCCD4a-1*, -2, -3, and -4 (Supplementary Fig. 1). This result indicates that these genes are members of a small gene family in the 'Paragon' genome. The protein coding sequence of *CmCCD4a-1* was identical to that of *CmCCD4a* previously reported by Ohmiya et al. (2006). *CmCCD4a-4* was determined to be a pseudogene, because in-frame stop codons caused by nucleotide deletions were found in the protein coding sequence (Supplementary Fig. 1).

Using RT-PCR-mediated cloning, we obtained 76 *CmCCD4a* cDNA clones from 'Paragon', 94 from '94-765', and 50 from PM, and sequenced all of them. The nucleotide sequences of each of the four *CmCCD4a* homologs among these three taxa were identical. *CmCCD4a-1* and *CmCCD4a-2* were the main genes expressed in 'Paragon' and PM (Table 2). In '94-765', 97% of the transcripts were *CmCCD4a-2*,

Table 2 Proportions of *CmCCD4a* homologs expressed in ray petals

	'Paragon'	'94-765' (WT)	'Pink Marble'
<i>CmCCD4a-1</i>	23 (30)	0 (0)	28 (56)
<i>CmCCD4a-2</i>	48 (63)	91 (97)	20 (40)
<i>CmCCD4a-3</i>	2 (3)	3 (3)	0 (0)
<i>CmCCD4a-4^a</i>	3 (4)	0 (0)	2 (4)
Total clones	76 (100)	94 (100)	50 (100)

Values indicate the numbers (%) of clones obtained by RT-PCR-mediated cloning

^a Pseudogene (see text for details)

and *CmCCD4a-1* was not detected. *CmCCD4a-3* was expressed at a low level in '94-765' but was not detected in PM.

The genomic sequence of *CmCCD4a-2* showed similar structural features to those previously reported for *CmCCD4a-1* (Ohmiya et al. 2006), such as an open reading frame of 1797 bp predicted to encode a 599-amino-acid polypeptide (identity 94%; similarity 97% to *CmCCD4a-1*) and a 97-bp intron 1442 bp downstream from the start codon (Supplementary Fig. 1). The sequence similarity of the promoter region between *CmCCD4a-1* and *CmCCD4a-2* was lower than that of coding region (identity 79%; similarity 93%). As observed in *CmCCD4a-1*

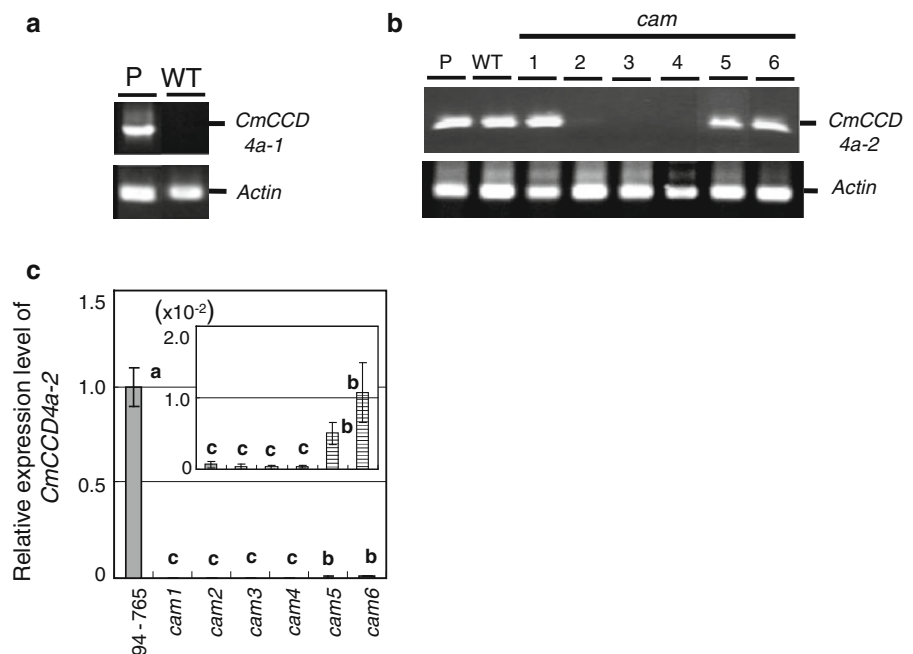
(Ohmiya et al. 2006), a predicted plastid-targeting peptide was identified in the N-terminal region of *CmCCD4a-2*, suggesting that both *CmCCD4a-1* and *CmCCD4a-2* are localized in the chromoplast.

Analyses of petal color mutants derived from '94-765'

To clarify the involvement of *CmCCD4a* in the level of carotenoids in ray petals, we used six independent petal color mutants (*cam1–cam6*) found among regenerants derived from '94-765' (Fig. 1a). Ray petals of the wild type of '94-765' (WT) contained $4.1 \pm 0.3 \mu\text{g/g}$ FW of carotenoids. Ray petals of *cam1–cam4* contained 6 to 8 times that level; those of *cam5* and *cam6* accumulated 1.3 to 1.8 times that level (Fig. 1b). Ray petals of *cam5* and *cam6* had lower anthocyanin contents than the WT (Supplementary Fig. 2a).

To evaluate whether genes encoding *CmCCD4a-1* and *CmCCD4a-2* exist in the genomes of the WT and each mutant, we designed primers specific to each homolog and performed genomic PCR. In the WT, a fragment of appropriate length was amplified with the *CmCCD4a-2* primer set (Fig. 2b), whereas no band was detected with the *CmCCD4a-1* primer set (Fig. 2a), indicating that the WT genome lacks an intact copy of *CmCCD4a-1*. The result was consistent

Fig. 2 Genomic PCR and RT-qPCR analyses of *CmCCD4a* in WT and *cam1–cam6*. **a** Genomic PCR with *CmCCD4a-1*-specific primers (Pro4a-1-F and Pro4a-1-R; Table 1). **b** Genomic PCR with *CmCCD4a-2*-specific primers (Pro4a-2-F and Pro4a-2-R; Table 1). Genomic DNAs of 'Paragon' (P), WT, and *cam1–cam6* were used as templates. An *actin* primer set (Actin-F and Actin-R; Table 1) was used as a PCR control. **c** Relative *CmCCD4a-2* expression level in ray petals. Error bar: \pm SEM ($n = 6$). A graph with expanded ordinate is shown in the inset



with that of the RT-PCR-mediated cloning, in which clones corresponding to *CmCCD4a-1* were not detected among the *CmCCD4a* clones from the WT (Table 2). Genomic PCR analysis of *cam1–cam6* showed that no intact copy of *CmCCD4a-2* was present in the genome of *cam2*, *cam3*, or *cam4*, but *CmCCD4a-2* was detected in the genomes of *cam1*, *cam5*, and *cam6* (Fig. 2b). The nucleotide sequences of *CmCCD4a-2* from 792 bp upstream of the start codon to the stop codon were identical among WT, *cam1*, *cam5*, and *cam6* (data not shown).

To investigate whether *CmCCD4a-2* was involved in the increase in carotenoid content in ray petals of the *cam* mutants, we examined the relationship between carotenoid content and the level of *CmCCD4a-2* expression. RT-qPCR analysis showed that the decrease in *CmCCD4a-2* expression level was

accompanied by an increase in the carotenoid content in ray petals of all six *cam* mutants (Figs. 1, 2c). Expression of *CmCCD4a-2* was not detected in *cam1–cam4*. The expression level of *CmCCD4a-2* in *cam5* and *cam6* was 0.5–1% of that in the WT. Expression of *CmCCD4a-3* was extremely low in ray petals of both the WT and the *cam* mutants (data not shown).

Analyses of ‘Marble’ cultivars

We analyzed ‘Marble’ cultivars, another type of petal color mutants, which were obtained from bud sports all tracing back to PM. The ‘Marble’ cultivars display a variety of ray petal colors, including white, yellow, orange, and purple, derived from combinations of anthocyanins and carotenoids (Figs. 3a, Supplementary Fig. 2b). The patterns and levels of carotenoids

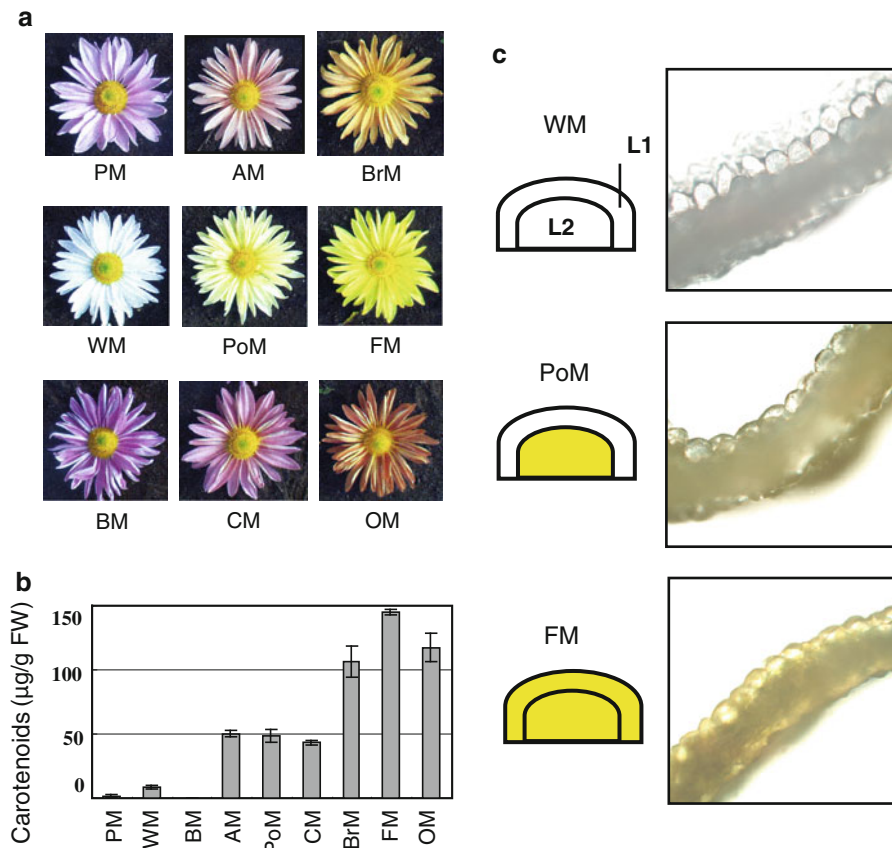


Fig. 3 Ray petal colors and carotenoid contents of ‘Marble’ cultivars. **a** Flowers of ‘Marble’ cultivars. Color variants are arranged horizontally from white to yellow and vertically from white to purple. **b** Carotenoid contents in ray petals. Error bar \pm SEM ($n = 3$). **c** Cross-sections of ray petals of WM, PoM, and

FM. Illustrations show carotenoid accumulation patterns in L1 and L2 of ray petals. AM ‘Apricot Marble’; BM ‘Blue Marble’; BrM ‘Bronze Marble’; CM ‘Coral Marble’; FM ‘Florida Marble’; OM ‘Orange Marble’; PM ‘Pink Marble’; PoM ‘Polished Marble’; WM ‘White Marble’

among ‘Marble’ cultivars fell into one of three categories: PM, WM, and BM, with no yellow in the petals; AM, PoM, and CM, which had yellowish L2 layers (Shibata and Kawata 1986) and contained approximately 50 $\mu\text{g/g}$ FW carotenoids; and BrM, FM, and OM, which had yellowish L1 and L2 layers and contained 100–150 $\mu\text{g/g}$ FW carotenoids (Fig. 3b). The purplish petal colors of BM, CM, and OM were due to high levels of anthocyanins (Supplementary Fig. 2).

In ray petals of PM, mainly *CmCCD4a-1* and *CmCCD4a-2* were expressed, and expression of *CmCCD4a-3* was not detected (Table 2). To evaluate whether *CmCCD4a-1* and *CmCCD4a-2* were involved in the variation in carotenoid content of ‘Marble’ cultivars, we performed genomic PCR and RT-qPCR

analyses. The genomic PCR analysis showed that *CmCCD4a-1* was present in the genomes of PM and AM (Fig. 4a). We could not detect it in WM, BM, PoM, CM, BrM, FM, or OM, indicating that these cultivars did not contain an intact copy of *CmCCD4a-1*. RT-qPCR showed that only PM expressed a high level of *CmCCD4a-1* among these ‘Marble’ cultivars (Fig. 4b). The expression level of *CmCCD4a-1* in AM was detectable but extremely low, and the levels in WM, BM, PoM, CM, BrM, FM, and OM were below the level of detection (Fig. 4b). The genomic PCR analysis showed that the gene encoding *CmCCD4a-2* existed in the genomes of all of the ‘Marble’ cultivars we examined (Fig. 4c). The RT-qPCR analysis showed that *CmCCD4a-2* was expressed in PM, WM, BM, AM, PoM, and CM but was not detected

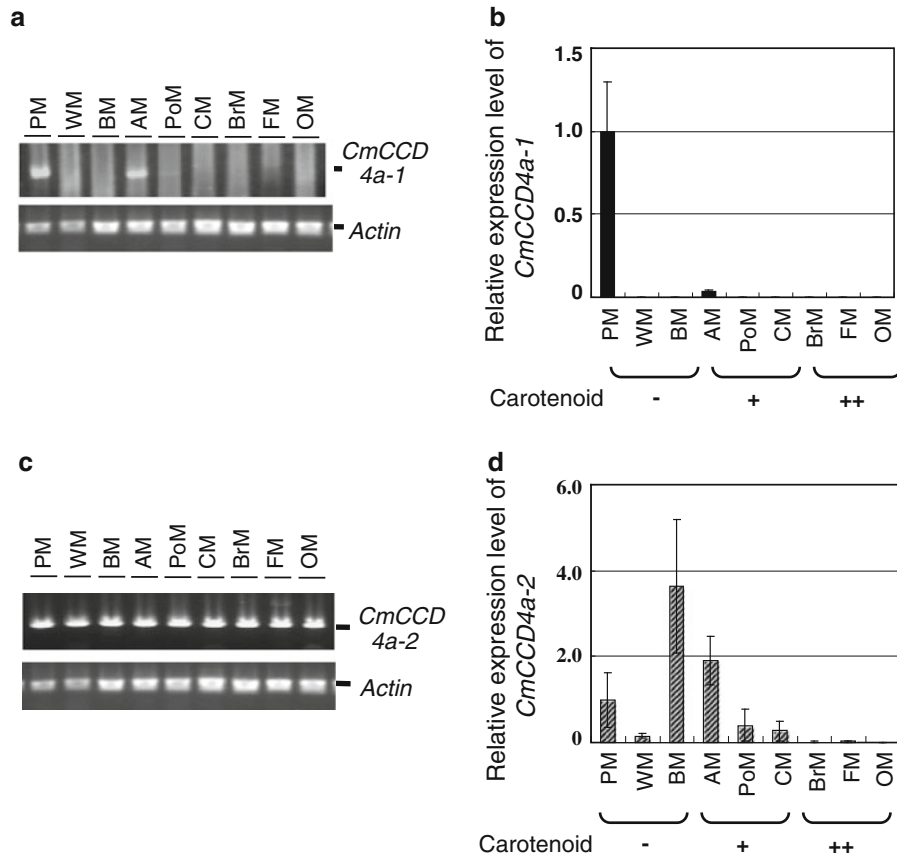


Fig. 4 Genomic PCR and RT-qPCR analyses of *CmCCD4a* in ‘Marble’ cultivars. **a** Genomic PCR with gene-specific primers for *CmCCD4a-1*. Genomic DNAs of ‘Marble’ cultivars were used as templates. An *actin* primer set was used as a PCR control. **b** Relative *CmCCD4a-1* expression level in ray petals. Error bars: \pm SEM ($n = 6$). **c** Genomic PCR with gene-specific

primers for *CmCCD4a-2* (Pro4a-2-F and Pro4a-2-R; Table 1). Genomic DNAs of ‘Marble’ cultivars were used as templates. **d** Relative *CmCCD4a-2* expression levels in ray petals. Error bars \pm SEM ($n = 6$). Abbreviations of cultivars are defined as for Fig. 3

in BrM, FM, or OM (Fig. 4d). Expression levels of *CmCCD4a-2* in ray petals of BM and AM were higher than that in ray petals of PM (Fig. 4d).

Discussion

We previously isolated a gene encoding *CmCCD4a* that was specifically expressed in white ray petals of chrysanthemum. Suppression of *CmCCD4a* expression by RNAi in white-flowered chrysanthemums produced yellow-flowered transformants (Ohmiya et al. 2006, 2009). In addition, experiments involving crosses between white- and yellow-flowered chrysanthemums showed that the presence of *CmCCD4a* led to the development of white ray petals (Yoshioka et al. 2010). From these results we hypothesized that *CmCCD4a* is involved in the regulation of carotenoid formation or accumulation in ray petals by cleaving carotenoids into colorless apocarotenoids. Here, we tested this hypothesis by analyzing the relationships between *CmCCD4a* expression and carotenoid content in petal color mutants of chrysanthemum.

Analyses of RT-PCR-mediated cloning followed by sequencing showed that *CmCCD4a* is actually a small gene family in the chrysanthemum genome. Four *CmCCD4a* homologs were identified in the clones obtained from ‘Paragon’; among these, *CmCCD4a-1* and *CmCCD4a-2* were the most highly expressed in ray petals. On the other hand, the laboratory line ‘94-765’ lacks an intact copy of *CmCCD4a-1* in the genome, and *CmCCD4a-2* was the most highly expressed in the ray petals. Using the petal color mutants *cam1*–*cam6*, which showed higher levels of carotenoid than their progenitor line ‘94-765’ (WT), we analyzed the relationship between the level of *CmCCD4a-2* expression and the carotenoid content in ray petals. A remarkable decrease in *CmCCD4a-2* expression occurred in ray petals of all six *cam* mutants (Fig. 2c), with a corresponding increase in carotenoid content (Fig. 1b). We found two different causes for the carotenoid accumulation in ray petals of *cam* mutants. In *cam2*, *cam3*, and *cam4*, *CmCCD4a-2* was lost from the genome or was no longer intact (Fig. 2b). In *cam1*, *cam5*, and *cam6*, *CmCCD4a-2* could be detected (Fig. 2b) but its expression was suppressed (Fig. 2c). Genomic sequences of *CmCCD4a-2* in *cam1*, *cam5*, and *cam6* were identical to that of the WT (data not shown). Therefore, this

decrease in expression was not caused by any sequence changes such as nucleotide substitutions, insertions, or deletions, but may have been caused by the loss of a function that normally promotes or enhances transcription of *CmCCD4a-2*.

As carotenoids accumulated in ray petals of both *cam5* and *cam6*, from 1.3 up to 1.8 times the level in the WT (Fig. 1b) even though the *CmCCD4a-2* expression level in the mutants was 1/100 or less (Fig. 2c), one explanation is that excess *CmCCD4a* is expressed in ray petals of the WT, and the levels in *cam* mutants, even though greatly reduced, are still high enough to cleave carotenoids. Another possibility is that the amounts of carotenoids synthesized in ray petals of both the WT and the *cam* mutants are lower than in other chrysanthemum cultivars (Kishimoto et al. 2004, 2007). A similar case was observed in ‘Sei-Marine’: even though *CmCCD4a* expression was reduced to 2% of wild type levels, only a small amount of carotenoid accumulated in ray petals (Ohmiya et al. 2006).

Chrysanthemum ray petals are composed of two cell layers, superficial (L1) and internal (L2) (Stewart and Derman 1970). Each layer is derived from a different somatic cell, whose genome could mutate independently. Because adventitious shoots develop from a single cell (Broertjes et al. 1976), the *cam* mutants obtained by somaclonal variation through tissue culture are not usually chimeras: any petal color mutation in the progenitor cell would be present in both L1 and L2. On the other hand, petal color mutants derived from bud sports, including ‘Marble’ cultivars, are often periclinal chimeras in which the color of each cell layer of ray petals may change independently (Bush et al. 1976; Boase et al. 1997; Shibata and Kawata 1986). In addition, PM, the original ‘Marble’ cultivar, contained intact copies of both *CmCCD4a-1* and *CmCCD4a-2*. The mechanism of petal color variation due to carotenoid accumulation in ‘Marble’ cultivars is, therefore, more complicated than that in the *cam* mutants derived from ‘94-765’.

Shibata and Kawata (1986) reported the carotenoid accumulation patterns in ray petals of ‘Marble’ cultivars, which we have illustrated in Fig. 5. In WM, PM, and BM, carotenoids do not accumulate in either L1 or L2. In FM, BrM, and OM, in contrast, they accumulate in both layers. In PoM, AM, and CM, they accumulate only in L2. OM was isolated as a bud sport from BrM (Shibata and Kawata 1986), but the

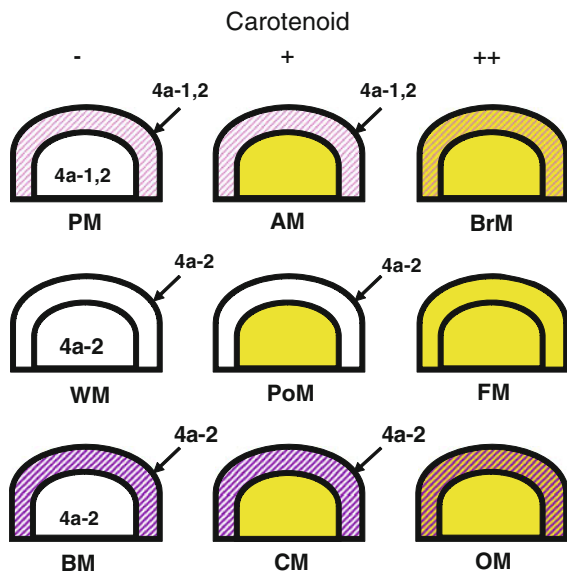


Fig. 5 Schematic diagrams of the chimeric pigment distribution in ray petals of ‘Marble’ cultivars, arranged as in Fig. 3a. Layer L1 covers layer L2. Carotenoid accumulation patterns (indicated by yellow) are based on the results described by Shibata and Kawata (1986). Expression patterns of *CmCCD4a-1* and *CmCCD4a-2* (indicated by “4a-1” and “4a-2”) are based on the results in this study. Anthocyanins (indicated by red stripes) accumulated only in L1 (data not shown). Abbreviations of cultivars are defined as for Fig. 3

breeding processes leading to the other ‘Marble’ cultivars are unknown. We hypothesize that petal color variation is caused by the loss of enzymatic activity of *CmCCD4a* in L2 of PoM, AM, and CM and in both L1 and L2 of FM, BrM, and OM. On the basis of the RT-qPCR analysis using primers specific to either *CmCCD4a-1* or *CmCCD4a-2*, we hypothesize that *CmCCD4a-1* and *CmCCD4a-2* are expressed in the histogenic layers of ray petals of ‘Marble’ cultivars as illustrated in Fig. 5. Because *CmCCD4a-1* expression cannot be detected in WM, BM, PoM, and CM (Fig. 4b), we hypothesize that *CmCCD4a-2* is expressed in the layers where carotenoids did not accumulate, i.e., in both L1 and L2 of WM and BM and in L1 of PoM and CM. Because both *CmCCD4a-2* and *CmCCD4a-1* were expressed in AM (Fig. 4b, d), and L2 contains carotenoid, we assume that the two *CmCCD4a* genes are expressed in L1. Considering the fact that PM is the original cultivar of AM and has higher expression of *CmCCD4a-1* than AM, we hypothesize that both *CmCCD4a-1* and *CmCCD4a-2* are expressed in L1 and possibly in L2 of PM.

Although the typical chromosome number of chrysanthemum is 54, the actual number varies widely with cultivar, ranging from 36 to 85 (Endo 1969; Shibata et al. 1998). In addition, some cultivars have odd numbers such as 53 or 55. This variety of karyotypes suggests that chrysanthemum is able to tolerate large-scale changes in genome composition. Dowrick and Bayoumi (1966) showed that changes in chromosome number and chromosome fragmentation are sometimes responsible for changes in petal color of chrysanthemum. Here, we show that some petal color mutants of chrysanthemum lost the activity (and sometimes the actual copy) of one or more *CmCCD4a* genes, which resulted in carotenoid accumulation. A stepwise decrease of *CmCCD4a-1* and *CmCCD4a-2* activity among ‘Marble’ cultivars appears to be correlated with a corresponding stepwise increase in carotenoid levels in ray petals. It is possible that there are several copies of *CmCCD4a-1* and *CmCCD4a-2* in the genome of PM. The phenotypes of the ‘Marble’ cultivars (Fig. 5) indicate that fewer steps are needed to eliminate *CmCCD4a* activity in L2 than in L1, suggesting that fewer copies (or fewer active copies) of *CmCCD4a* exist in L2 than in L1. Independent mutation in each cell layer may also cause stepwise decreases in the amount of *CmCCD4a*.

There are some examples in which copies of *CmCCD4a-1*, *CmCCD4a-2*, or both were present in the genome but were not expressed in ray petals: for example, *CmCCD4a-2* in BrM, FM, OM, and *cam1*. On the other hand, expression of *CmCCD4a-2* was higher in BM and AM than in the progenitor (PM). An increase in expression of a gene encoding CCD4 was also reported in a fruit-color mutant of peach that showed white flesh (Brandi et al. 2011). These examples might be caused by the loss of functions that enhance or suppress transcription of *CCD4*. However, a transcriptional regulator that controls *CmCCD4a* expression has not been discovered yet. Further study will be needed to explain these expression patterns.

The enzymatic activities of CCDs are still a matter of debate. Floss et al. (2008) reported different substrate specificities of CCD1 between in vitro and in planta analyses. In vivo analysis using carotenoid-accumulating *Escherichia coli* cells showed that recombinant *CmCCD4a* (identical to *CmCCD4a-1* in this study) specifically cleaves double bonds at the 9,10 (9',10') positions of β -carotene and zeaxanthin

(Huang et al. 2009). It does not, however, cleave xanthophylls, including zeaxanthin and lutein, in vitro, but it does cleave the double bond at the 9,10 position of 8'-apo- β -caroten-8'-al. However, in planta activity of CCD4 has not yet been elucidated. The carotenoids in ray petals of *cam2* (which lost *CmCCD4a-2*) consist mainly of lutein derivatives (Supplementary Fig. 3), as in other chrysanthemum cultivars previously reported (Kishimoto et al. 2004). The results suggest that *CmCCD4a-2* cleaves lutein derivatives as substrates in planta.

Conclusion

We show that loss of *CmCCD4a*(s) or transcriptional regulator(s) occurred during the process of petal color mutation in chrysanthemum. These events resulted in decreased *CmCCD4a* expression and increased carotenoid levels in ray petals, indicating that members of the *CmCCD4a* family play a critical role in regulating carotenoid accumulation. We thereby propose that *CmCCD4a* genes represent the single dominant gene that inhibits carotenoid accumulation predicted in earlier studies (Stewart and Derman 1970; Jordan and Reinmann-Philipp 1983; Hattori 1991; Boase et al. 1997).

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