“Yellow Jimba”: Suppression of Carotenoid Cleavage Dioxygenase (*CmCCD4a*) Expression Turns White Chrysanthemum Petals Yellow

Akemi Ohmiya*, Katsuhiko Sumitomo and Ryutaro Aida

National Institute of Floricultural Science, Tsukuba, Ibaraki 305-8519, Japan

‘Jimba’ is the most popular white-flowered chrysanthemum cultivar in Japan. A yellow-flowered cultivar with the same growth properties as ‘Jimba’ will benefit growers because both forms could be produced under the same conditions. Many breeders have therefore tried to produce a “Yellow Jimba” by mutation breeding but have not yet succeeded. Previously, we showed that color mutation from white to yellow in the petals of ray florets is caused by the loss of a carotenoid cleavage dioxygenase gene, *CmCCD4a*. Here we introduced two separate *CmCCD4a* RNAi constructs into ‘Jimba’ by *Agrobacterium*-mediated transformation. The double-transformation effectively suppressed *CmCCD4a* expression in petals, which became yellow. The yellowest transformants contained 102 $\mu\text{g}\cdot\text{g}^{-1}$ FW carotenoids in the petals, and the expression level of *CmCCD4a* was 0.4% of the wild-type level. Although the transformed plants were significantly smaller than the wild type, flower size was unchanged. We consider that *CmCCD4a* could become a powerful tool for petal color manipulation from white to yellow.

Key Words: *Agrobacterium*-mediated transformation, carotenoid, carotenoid cleavage dioxygenase (*CmCCD4a*), *Chrysanthemum morifolium* Ramat., flower color.

Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the most important ornamental flowers in the world and has been bred for more than 2000 years. Flowers come in a variety of colors, including white, yellow, orange, and purple-red. In Japan, white-flowered cultivars are the most popular, followed by yellow-flowered (Shibata, 1994), and are used mainly for funerals. ‘Jimba’ is the most popular white-flowered cultivar, accounting for about 30% of total chrysanthemum production.

Mutation breeding, such as by bud sport selection or ion-beam radiation, sometimes produces a white-to-yellow petal color mutation (Boase et al., 1997; Machin and Scopes, 1978). Efforts have been made by mutation breeding to breed yellow-flowered cultivars with the same growth properties as ‘Jimba’, but breeding a “Yellow Jimba” has not yet succeeded.

The yellow color of chrysanthemum petals originates from carotenoids (Kishimoto et al., 2004). Although white-flowered cultivars do not contain carotenoids in

their petals, most genes encoding carotenoid biosynthesis enzymes are expressed, suggesting that carotenoids are synthesized in white petals (Kishimoto and Ohmiya, 2006). In a previous study (Ohmiya et al., 2006), we screened gene transcripts in white and yellow petals of ray florets to search for the factor regulating carotenoid accumulation in chrysanthemum petals, and we identified a clone specifically expressed in white petals. The deduced amino acid sequence was highly homologous to carotenoid cleavage dioxygenase (designated *CmCCD4a*). Suppression of *CmCCD4a* expression in the white-flowered cultivar ‘Sei-Marine’ by RNA interference (RNAi) resulted in yellow petals. This result shows that the carotenoids are synthesized in white petals but are subsequently degraded into colorless compounds by *CmCCD4a*. We anticipate that *CmCCD4a* could become a powerful tool for petal color manipulation.

Here, we introduced RNAi constructs of *CmCCD4a* into ‘Jimba’ with the aim of making yellow petals; we compared the differences in carotenoid contents, levels of *CmCCD4a* expression, and growth between wild-type and RNAi plants. We showed that double-transformation of RNAi constructs effectively suppressed the target gene and produced a yellow-flowered ‘Jimba’.

Received; January 23, 2009, Accepted; March 6, 2009.

* Corresponding author (E-mail: ohmiya@affrc.go.jp).

Materials and Methods

Plant material

Chrysanthemum cultivar ‘Jimba’ was used for the experiments. Leaf tissues for transformation were obtained from plants grown *in vitro* in Murashige–Skoog medium with half-strength minerals (1/2 MS) (Murashige and Skoog, 1962) solidified with 0.2% (w/v) gellan gum; the plants were grown at 25°C under a 16-h light:8-h dark photoperiod under fluorescent light (photon flux density 70 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$).

RNAi constructs and *Agrobacterium*-mediated transformation

We introduced two separate RNAi constructs into ‘Jimba’ by *Agrobacterium*-mediated transformation. In the first transformation, we used an existing *CmCCD4a* RNAi construct with a kanamycin resistance gene (designated pEF1 α ::S-AS, Fig. 1a; Ohmiya et al., 2006). In the second transformation, we created a different *CmCCD4a* RNAi construct with a hygromycin resistance gene (designated p35S::AS-Cm^r-S, Fig. 1b) using the Gateway system (Invitrogen, Carlsbad, CA, USA) as follows. A 343-bp *CmCCD4a* fragment containing an *attB2* sequence at the 5'-end and an *attB1* sequence at the 3'-end was amplified by polymerase chain reaction (PCR) using the forward primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGCTCTTTTCCACATCTCTTC-3' and the reverse primer 5'-GGGGACAAGTTTGTACAAAAGCAGGCTCATATTTGGGGTCAACCGAAAC-3'. This fragment contained a different part of the coding region of *CmCCD4a* used for pEF1 α ::S-AS. It was cloned into the pENTR221 vector (Invitrogen) by the BP reaction using Gateway BP clonase II enzyme mix (Invitrogen), and then subcloned into the binary vector pH7GWIWG2(II) (VIB, Ghent, Belgium) by the LR reaction using Gateway LR clonase II enzyme mix (Invitrogen), as described in the manufacturer's instructions.

Transgenic plants were obtained by the *Agrobacterium*-mediated transformation system, as described by Aida et al. (2004).

Analysis of carotenoid contents

Tissues (0.5 g) were ground in acetone, and the extracts were partitioned between diethyl ether and aqueous NaCl. Absorbance of the diethyl ether layer was measured at a wavelength of 445 nm. Carotenoid content was calculated using the $E_{1\%}^{1\text{cm}}$ value of lutein (Britton, 1995) and expressed as micrograms of lutein equivalent per gram fresh weight (g^{-1} FW) of tissue.

Analysis of *CmCCD4a* expression

Total RNA was extracted from the petals of the outermost part of the ray florets and treated with DNaseI using an SV Total RNA Isolation System (Promega, Madison, WI, USA). cDNAs were synthesized from total RNA using a Superscript First-Strand Synthesis System (Invitrogen). Levels of *CmCCD4a* transcripts were analyzed by real-time reverse transcription (RT)-PCR as described by Ohmiya et al. (2006).

Genomic PCR analysis of transgenes

Genomic DNA was extracted from leaves using the cetyl trimethyl ammonium bromide method (Murray and Thompson, 1980). Approximately 50 ng DNA was used as a template for PCR. The primers used to amplify the neomycin phosphotransferase II (NPTII) coding sequence in pEF1 α ::S-AS and the hygromycin phosphotransferase (HPT) coding sequence in p35S::AS-Cm^r-S are as follows: NPTII forward, 5'-CAAGATGGATTG CACGCAGG-3'; NPTII reverse, 5'-GAAGAACTCGT CAAGAAGGCG-3'; HPT forward, 5'-CGACGTCT GTCGAGAAGTTTC-3'; and HPT reverse, 5'-CACG GCCTCCAGAAGAAGA-3'. PCR products were separated on a 1.0% agarose gel and visualized by ethidium bromide staining.

Fig. 1. Schematic diagram of the RNAi constructs used to suppress *CmCCD4a* expression in ‘Jimba’. Only the regions between right and left borders are shown. (A) pEF1 α ::S-AS; (B) p35S::AS-Cm^r-S. S1 and S2, *CmCCD4a* fragment in sense orientation; AS1 and AS2, *CmCCD4a* fragment in antisense orientation; PEF1 α , tobacco elongation factor 1 α promoter; P_{35S}, cauliflower mosaic virus 35S promoter; PNAS, nopaline synthase promoter; TNOS, nopaline synthase poly(A) addition sequence; HPT, hygromycin phosphotransferase II coding sequence; NPT, neomycin phosphotransferase II coding sequence; Cm^r, chloramphenicol acetyltransferase coding sequence. The lengths of S1, AS1, S2 and AS2 are 442 bp, 293 bp, 343 bp and 343 bp, respectively.

Analysis of growth properties

On 8 August, cuttings (4 cm) with 3 or 4 leaves of both wild-type and RNAi plants were placed in plug-cell trays filled with commercial horticultural soil (Metro-Mix 360; Sun Gro Horticulture, Bellevue, WA, USA). On 26 August, 12 rooted cuttings of each were planted individually in 12-cm plastic pots containing commercial horticultural soil (Kureha-Engei-Baido; Kureha Chemical Co. Ltd., Tochigi, Japan). Plants were grown in a closed greenhouse (maintained at an air temperature above 18°C and ventilated above 25°C) under a natural photoperiod plus a 4-h night break from 2200 to 0200 h with incandescent lamps ($3.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; K-RD, 100 V, 60 W; Panasonic Electric Works Ltd., Osaka, Japan). Liquid fertilizer (100 mg NPK·L⁻¹) was administered twice a week. On 16 September, all plants were transferred to a closed greenhouse under flower-inducing short-day conditions (<12.0 h). Stem length and the number of unfolded leaves were measured. Axillary shoots were removed and plants were grown as single-stem plants. When the flowers were at full bloom, we measured the stem length, flower diameter, length and width of leaves at the 10th node below the apical flower, and counted the ray florets and disk florets.

Statistical analysis

Growth properties of wild-type and RNAi plants were compared by the Tukey-Kramer test at the 5% level.

Results

Suppression of *CmCCD4a* expression by RNAi

We obtained 61 independent transformed 'Jimba' plants containing the first RNAi construct. Although the carotenoid content of petals at the outermost part of the ray florets was below the detection limit in all transformants, some plants had pale yellow petals around the disk florets (Fig. 2a). We then chose a transformant (designated T₁) with the yellowest petals for the second transformation with the second RNAi construct. We obtained 50 independent double-transformed plants, more than half of which had yellow petals. We chose four plants (designated T₂-1, -2, -3, and -4) for further analysis. Petals from the yellowest flower (T₂-2) contained up to $102 \mu\text{g g}^{-1}\cdot\text{FW}$ of carotenoids (Fig. 2b). Integration of the transgene into the genome of transformants was confirmed by PCR analysis. Fragments of the predicted size of NPTII were amplified in both T₁ and T₂ plants, and those of HPT were amplified only in T₂ plants (Fig. 3).

Real-time RT-PCR analysis of *CmCCD4a* transcripts in petals showed a high level of *CmCCD4a* transcripts

Fig. 2. Analyses of *CmCCD4a* expression and carotenoid contents in petals of wild-type (WT) and single- (T₁) and double-transformed (T₂) 'Jimba' plants. (A) Flowers of wild-type and single- and double-transformed 'Jimba'. (B) Carotenoid concentrations in petals of outermost part of ray florets. Mean $\pm$ SE, $n=3$. (C) Levels of *CmCCD4a* expression. Quantitative real-time RT-PCR analyses were performed using primer sets specific for *CmCCD4a*. The abundance of *CmCCD4a* mRNA relative to values of wild-type plants is shown after normalization to that of *actin*. Mean $\pm$ SE, $n=3$.

Fig. 3. Genomic PCR analysis of transgenes in wild-type (WT) and single- (T₁) and double-transformed (T₂) ‘Jimba’ plants. PCR was performed using primer sets specific for either NPTII or HPT. DNA plasmids pEF1 α ::S-AS (P1) and p35S::AS-Cm^r-S (P2) were used as positive controls.

in wild-type plants, at about the same level observed in petals of other white-flowered cultivars such as ‘Paragon’ and ‘White Marble’ (Ohmiya et al., 2006). The level of *CmCCD4a* expression in petals of T₁ was only 16.4% that of the wild type (Fig. 2c). *CmCCD4a* expression was further suppressed by double-transformation of RNAi constructs to 0.4%–8.0% of the wild type.

Growth properties of RNAi transformants

Wild-type, T₁, and T₂ plants were grown as single-stem plants and their growth properties were compared (Fig. 4 and Table 1). On transfer to short-day conditions, RNAi plants showed a slight reduction in stem length compared with the wild type. The reduction became more pronounced at full bloom: 7% reduction in T₁ plants and 10%–16% in T₂. The length and width of leaves of T₂ plants were also significantly smaller than those of the wild type. T₁ plants showed no difference in days to flower from the wild type, but some T₂ plants flowered earlier. There was no significant difference in the diameter of flowers between wild-type and RNAi plants, but RNAi plants had significantly more disk florets. There was no difference in the number of leaves on transfer to short-day conditions, and little difference in the number of new leaves after transfer to short-day conditions; therefore, the number of axillary buds was unchanged after transformation, but when axillary buds initiated shoot growth, T₂ plants produced significantly fewer axillary shoots than the wild type.

Discussion

Petal color mutation is common in chrysanthemum, whether it be spontaneous or induced (Boase et al., 1997; Machin and Scopes, 1978). Many chrysanthemum cultivars have arisen by mutation breeding (van Hartena and Broertjes, 1989). Petal color mutation follows a distinct pattern: white-flowered cultivars can mutate to yellow, but yellow-flowered cultivars do not mutate to white (Broertjes, 1966). This directional pattern of mutation was supposed to indicate loss of a chromosomal region or a single gene (Dowrick and El-Bayoumi, 1966). Experiments in which white- and yellow-flowered chrysanthemums were crossed showed that the white petal color is dominant over yellow, suggesting that a single dominant gene inhibits carotenoid accumulation

Fig. 4. Wild-type (WT) and single- (T₁) and double-transformed (T₂) ‘Jimba’ plants.

(Hattori, 1991; Langton, 1980; Teynor et al., 1989). Recently, we demonstrated that the petal color mutation from white to yellow is caused by loss of the *CmCCD4a* gene (Ohmiya et al., 2006). In white petals, carotenoids are synthesized but then degraded by *CmCCD4a*. Loss of enzyme activity of *CmCCD4a* results in the accumulation of carotenoids, so the petals grow yellow.

Genetic transformation by *Agrobacterium* is a useful method for inserting a new trait into economically important crops and ornamental plants; its main advantage is the ability to introduce favorable mutations of an outstanding cultivar without altering the remaining genotype. In some crops, carotenoid content and composition have been genetically manipulated by introducing genes related to carotenogenesis to improve nutritional value (see reviews by Fraser and Bramley, 2004; Tanaka and Ohmiya, 2008); however, few data are relevant to altering petal color by genetic manipulation of carotenoid metabolism. We have previously shown that the petal color of the chrysanthemum cultivar ‘Sei-Marine’ changed from white to yellow by the introduction of a *CmCCD4a* RNAi construct (Ohmiya et al., 2006). The result showed that *CmCCD4a* is a powerful tool for altering petal color from white to yellow or *vice versa*. We therefore tried to create a yellow-flowered ‘Jimba’ by suppressing

Table 1. Growth properties of wild-type and transformed 'Jimba' plants.

	WT	T ₁	T ₂₋₁	T ₂₋₂	T ₂₋₃	T ₂₋₄
Days to full bloom after short-day treatment	47.6±0.3 a	47.3±0.3 a	45.7±0.3 bc	45.4±0.3 c	46.7±0.2 b	47.1±0.3 a
Length of stems on transfer to short-day conditions (cm)	26.8±0.5 a	25.3±0.5 abc	23.7±0.6 c	25.4±0.4 abc	24.1±0.4 bc	26.0±0.5 ab
Length of stems at full bloom (cm)	72.0±0.6 a	67.0±0.5 b	60.1±0.8 e	61.7±0.7 de	63.4±0.8 cd	65.8±0.9 bc
Number of axillary bud outgrowth	21.1±1.1 a	22.0±1.0 a	18.5±1.5 ab	16.9±0.8 b	15.2±0.8 b	14.7±0.5 b
Number of leaves on transfer to short-day conditions	20.1±0.4 a	20.6±0.3 a	20.8±0.4 a	21.1±0.2 a	19.9±0.3 a	19.9±0.4 a
Increased number of leaves after transfer to short-day conditions	22.3±0.2 a	22.0±0.2 ab	21.6±0.2 b	22.3±0.3 ab	22.0±0.3 ab	22.9±0.3 ab
Length of leaf brades + petioles (mm)	107.6±1.4 a	107.0±1.7 ab	102.4±1.3 abc	100.9±1.5 bc	100.5±1.9 bc	99.4±1.6 c
Width of leaf brades (mm)	52.4±0.6 a	50.8±0.9 ab	47.5±1.1 bc	45.6±0.9 c	47.9±1.0 bc	47.8±1.3 bc
Diameter of flowers (mm)	133.2±1.6 a	133.1±1.1 a	131.7±2.0 a	133.8±1.6 a	129.3±1.1 a	132.8±1.0 a
Number of ray florets	160.2±2.9 ab	147.5±5.6 bc	136.1±5.4 c	145.0±5.4 bc	169.9±5.4 a	159.5±6.0 ab
Number of disc florets	92.4±5.1 a	133.7±6.5 b	174.6±6.7 c	139.9±5.5 b	115.4±5.4 ab	133.7±6.5 b

Different letters within a row indicate significant differences by the Tukey-Kramer Test ($P < 0.05$).

CmCCD4a expression. First, we introduced a *CmCCD4a* RNAi construct, pEF1 α ::S-AS, into 'Jimba', but none of the 61 transformants had notably yellow petals. Although the expression of *CmCCD4a* was reduced to 16.4% of the wild-type level, it seems that significant *CmCCD4a* enzyme activity still occurred. We therefore inserted another *CmCCD4a* RNAi construct, p35S::AS-Cm⁺-S, into a T₁ plant instead of scaling-up the single transformation of the first RNAi construct. This double-transformation was remarkably successful at suppressing the target gene. Among 50 transformants obtained, more than half had yellow petals. *CmCCD4a* expression was suppressed to as little as 0.4% of the wild-type level. The amount of carotenoids ($\leq 102 \mu\text{g}\cdot\text{g}^{-1}$ FW) in the petals of T₂ plants was still much less than that of yellow-flowered cultivars (Kishimoto et al., 2007), so if the expression of *CmCCD4a* could be completely knocked out, the yellow petal color might become much deeper.

RNAi is widely used as a sequence-specific method to knock down the expression of the target gene (see reviews by Kusaba, 2004; Small, 2007). This method affords considerable advantages over sense and antisense suppressions owing to its higher gene-silencing efficiency. Few data are available, however, as to the efficiency of the double introduction of RNAi constructs to substantially suppress the target gene. With regard to our double-transformation experiments, it is unclear why the second transformation suppressed the expression of *CmCCD4a* more effectively than the first transformation. Several differences were observed in the 2 RNAi constructs used in the experiments, namely, those in the promoters, trigger sequences, and the length of loop-forming sequences, which may have affected the efficiency.

Yellow-flowered mutants generally have lower vigor, with reduced height and flower size, than their parental white-flowered cultivars (Dowrick and El-Bayoumi, 1966). In addition, products of carotenoid cleavage play an important role in controlling branching (Schwartz et al., 2004). It is therefore important to examine the growth properties of plants with genetically manipulated

carotenoid metabolism. The vegetative tissues tended to be smaller in our RNAi plants. These differences might have been caused by somaclonal variation arising in tissue culture (Skirvin, 1978), or the compounds produced by enzymatic activity of *CmCCD4a* might affect the growth of transformants. In *Arabidopsis*, the carotenoid cleavage dioxygenases *AtCCD7* and *AtCCD8* are involved in the synthesis of a carotenoid-derived signaling molecule that is necessary for the regulation of lateral branching (Schwartz et al., 2004). In petunia (*Petunia hybrida*), RNAi of *PhCCD8*, a homolog of *AtCCD8*, resulted in increased branching (Snowden et al., 2005). Both *PhCCD8* and *CmCCD4a* RNAi plants were significantly shorter. In contrast to RNAi of *PhCCD8*, RNAi of *CmCCD4a* reduced the number of axillary bud outgrowth. Recently, the carotenoid-derived signaling molecule was identified as strigolactone (Gomez-Roldan et al., 2008). Because the compounds produced by the enzymatic activity of *CmCCD4a* have not been determined yet, further study will be needed to clarify whether the suppression of carotenoid cleavage in petals affects vegetative growth. Although RNAi plants were significantly shorter than wild-type plants, increasing the period of growth under night-break conditions before flower initiation by short-day treatment can compensate for this and produce plants tall enough for cut flowers, because the height of RNAi plants reached over 100 cm without flower initiation under night-break conditions (data not shown). We consider that suppression of *CmCCD4a* expression by RNAi offers a shorter route to the alteration of petal color from white to yellow in cultivars that have petal color resistant to alteration by mutation breeding.

Flower color is one of the most important factors determining the commercial value of a plant. Many efforts have therefore been undertaken to develop a wide range of flower colors in the breeding of ornamental plants; however, most plant species have limited flower color variation and lack particular colors. For example, morning glory (*Ipomoea nil*), *Eustoma*, and petunia lack yellow-flowered cultivars. If the mechanism for white

color formation in such plants is the same as in chrysanthemum, *CCD* will be a useful tool for introducing yellow petal color into these plants. It is also possible to change the petal color from yellow to white by overexpressing *CCD*. Because carotenoids are essential compounds for plants and play an important role in photoprotection, overexpression of *CCD* should be restricted to petals.

Acknowledgements

We thank Kiyomi Shimizu, Yumi Ohtsuka, Misako Takahashi and Miyuki Mori for technical assistance. This work was supported by grants from the National Agriculture and Food Research Organization (NARO), Japan.

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