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Significance of *CmCCD4a* orthologs in apetalous wild chrysanthemum species, responsible for white coloration of ray petals

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Abstract Most chrysanthemum (*Chrysanthemum morifolium* Ramat.) flowers have a central capitulum, composed of many disc florets that is surrounded by ray petals. *CmCCD4a*, a gene that encodes a carotenoid cleavage dioxygenase (CCD), is expressed specifically in the ray petals of chrysanthemum cultivars, and its expression leads to white ray petals as a result of carotenoid degradation. Here, we show that wild chrysanthemums with white ray petals have *CmCCD4a* orthologs, whereas those with yellow ray petals lack these orthologs, as is the case in chrysanthemum cultivars. *CmCCD4a* orthologs also exist in some lines of *Chrysanthemum pacificum* and *Chrysanthemum shiwogiku*, even though these species lack ray petals. Interspecific hybridization between *C. shiwogiku* and a yellow-flowered chrysanthemum cultivar showed that the *CmCCD4a* orthologs from *C. shiwogiku* lead to the development of white ray petals. This indicates that the translation products of the *CmCCD4a* orthologs maintain enzymatic activity that can degrade carotenoids in chrysanthemums, irrespective of whether or not the ray petals that *CmCCD4a* expression actually occurred.

Keywords Apetalous flower · Carotenoid cleavage dioxygenase · *C. shiwogiku* · *C. pacificum* · Interspecies crossing · Wild chrysanthemum

Abbreviations
CCD Carotenoid cleavage dioxygenase
RNAi RNA interference

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Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is an important ornamental species around the world, and flowers with a range of petal colors (such as white,

pink, purple, and pale yellow to deep orange) have been developed. Kishimoto et al. (2004, 2007) reported that, in chrysanthemum cultivars, the yellow color of the ray petals is derived from the presence of yellow carotenoids, mainly lutein derivatives, and the orange color is derived from a mixture of red anthocyanins and yellow carotenoids. The petal color has generally evolved to attract insects, birds, and other pollinators (Paige and Whitham 1985), so petal color is an important element in the plant reproduction. In addition, these characteristics are important determinants of the commercial value of the flowers in horticultural markets.

The existence of a dominant gene that suppresses carotenoid biosynthesis was suggested by Hattori (1991), but subsequent research demonstrated that the carotenoid biosynthesis pathway works almost equally well at the transcriptional level, both in plants with white ray petals and those with yellow ray petals (Kishimoto and Ohmiya 2006). Recently, a candidate gene was identified from a white-flowered chrysanthemum cultivar (Ohmiya et al. 2006). The gene shows high similarity to a gene for carotenoid cleavage dioxygenase (CCD), designated *CmCCD4a*. *CmCCD4a* is expressed specifically in the ray petals of chrysanthemum cultivars. Severe repression of *CmCCD4a* transcription by means of RNA interference (RNAi) has been shown to convert white ray petals into yellow ray petals in the transformants (Ohmiya et al. 2006). Thus, *CmCCD4a* expression degrades carotenoids into colorless compounds in the ray petals of cultivars that contain this gene, resulting in white ray petals.

Nine genes in the *CCD* family are found in the *Arabidopsis* genome, and the products of most of these genes have been demonstrated to perform enzymatic roles and to modify the physiological functions of their apocarotenoid products (Tan et al. 2003; Schwartz et al. 2004; Schmidt et al. 2006; see review: Auldridge et al. 2006). However, the biological function of *AtCCD4*, which shows about 61% identity to *CmCCD4a*, remains totally unknown. Recently, it has been shown that recombinant CCD4 proteins in different plant species cleave (apo)-carotenoids at the 9,10 (9′,10′) positions to yield β-ionone (Rubio et al. 2008; Huang et al. 2009). It is interesting to note that AtCCD4 and CmCCD4a have different substrate specificity: CmCCD4a cleaves β-carotene to produce β-ionone, whereas AtCCD4 does not exhibit enzymatic activity against β-carotene but exhibits enzymatic activity against 8′-apo-β-carotene-8′-al. This finding suggests that AtCCD4 and CmCCD4a have different biological functions.

Because wild chrysanthemums have some useful characteristics that chrysanthemum cultivars lack, and can interbreed with chrysanthemum cultivars, they are often used as breeding parents (Jong and Rademaker 1989, Tanikawa et al. 2006). Modern chrysanthemum cultivars are thought to have originated from hybrids between white- and yellow-flowered wild chrysanthemums (Dai et al. 2005). It is reasonable to assume that wild species and chrysanthemum cultivars with white ray petals possess the same mechanism for causing white ray petal coloration, and that this mechanism may be derived from the degradation of carotenoids.

Generally, chrysanthemums form compound flowers that are composed of many disc florets, which possess both pistils and stamens, at the center of the flower and an outer ring of ray petals. The capitulums of *Chrysanthemum pacificum* Nakai and *Chrysanthemum shiwogiku* Kitam. are composed of only the disc florets, and lack the ray petals. It is unknown whether they have *CmCCD4a* orthologs because they lack the ray petals in which *CmCCD4a* is specifically expressed.

In this paper, we report the results of a study that demonstrate that white-flowered wild chrysanthemums have *CmCCD4a* orthologs, and that the translated products of these orthologs contribute to the development of white ray petal coloration in wild species, as is the case for *CmCCD4a* in chrysanthemum cultivars. Furthermore, we found that the apetalous wild species also have functional *CmCCD4a* orthologs that are responsible for carotenoid degradation, independent of whether they form ray petals.

Materials and methods

Plant materials

Wild chrysanthemum species and the yellow-flowered chrysanthemum (*C. morifolium*) cultivar ‘Squash’ were obtained from the NIAS Genebank Project of the National Institute of Agrobiological Science (Tsukuba, Ibaraki, Japan). We used 10 wild species with white ray petals: *C. wakasaense* line 2-12,

C. ornatum line 4-11, *C. crassum* line 5-21, *C. zawadskii* line 8912, *C. weyrichii* line 8913, *C. zawadskii* var. *latilobum* line 10, *C. yoshinagantum* line 12, *C. japonense* line 13, *C. makinoi* line 18 and *C. yezoense* line 19, two apetalous wild species that lacked ray petals: *C. shiwogiku* line 4-8, *C. shiwogiku* line 8808, *C. pacificum* line 11 and *C. pacificum* line 8706, and two wild species with yellow ray petals: *C. indicum* line 2-3 and *C. seticuspe* line 1.

Southern blot analysis

Genomic DNA was isolated from chrysanthemum mature leaves using the cetyl trimethyl ammonium bromide method (Murray and Thompson 1980). *Bam*HI-digested genomic DNA (25 μg) was electrophoresed in 0.7% agarose gel and blotted onto Hybond-N+ nylon membrane (GE Healthcare Biosciences) using 20× SSC, followed by baking at 80°C for 2 h. The probe was prepared using PCR digoxigenin probe synthesis kit (Roche Diagnostics). Based on the sequence of *CmCCD4a* from the chrysanthemum cultivar 'Paragon', the following primer sequences were designed: 5′-GCAGACCC TAGGAAGGTTGCAC-3′ (1117-F), 5′-AGATTCCG GATTGAAAGAGGGTACC-3′ (1370-R). The filter was hybridized with a digoxigenin-labeled *CmCCD4a* probe and washed twice with 2× SSC plus 0.1% SDS at room temperature for 15 min, and then twice with 0.1× SSC plus 0.1% SDS at 68°C for 15 min. Luminescence from the reaction between anti-digoxigenin-alkaline phosphatase Fab fragments and CDP-Star (Roche Diagnostics) was detected using the Light-Capture AE-6962C (ATTO).

Interspecies crossing between apetalous wild species and 'Squash'

We chose two apetalous wild species, *C. shiwogiku* (line 4-8) and *C. pacificum* (line 8706), and the cultivar 'Squash', which was chosen as a representative of yellow-flowered cultivars because it lacks the *CmCCD4a* gene. Interspecies crosses were performed between both wild species and 'Squash'. Four combinations between the ovary parent and the pollen parent were produced: *C. shiwogiku* line 4-8 × 'Squash', *C. pacificum* line 8706 × 'Squash', 'Squash' × *C. shiwogiku* line 4-8, and 'Squash' × *C. pacificum* line 8706. These crossings produced 51, 22, 23, and five F_1 plants, respectively.

Colorimetry of F_1 progenies and scatterplots of color values

The color values for the ray petals of each F_1 plant were measured using a CM-2600d spectrophotometer (Konica Minolta), with values provided in the L*a*b* color space. The values of the a* and b* color components were plotted on the x- and y-axes, respectively, of scatterplots created using Excel software (Microsoft).

Genomic PCR using DNA from each progeny

CmCCD4a orthologs were detected by means of PCR using 10 ng of genomic DNA from the progenies as templates, and Advantage 2 DNA polymerase (Clontech) with 5% dimethyl sulfoxide in 1× solution of the reaction buffer provided by the manufacturer. The PCR procedure was as follows: 95°C for 2 min for initial denaturation, followed by 40 cycles of 95°C for 20 s, 65°C for 20 s, and final phase at 72°C for 1 min. The following primer sequences were used for this PCR reaction: 5′-CGGAGATACTATTGTGAT GGTGGCG-3′ (CCD4a-1245F); 5′-AATAATCAAA GCGTTGTTAGGTATT-3′ (CCD4a-1805R). For the PCR control, the actin gene was amplified using the following primer set: 5′-CTTGCGTTTGGATCTTG CTGGTCGTGA-3′ (Actin-291F), and 5′-AGCAGCT TCCATCCCAATCATAGACGG-3′ (Actin-556R).

Results and discussion

Ohmiya et al. (2006) reported that *CmCCD4a* was found in the genome of all examined white-flowered cultivars, whereas most of the yellow-flowered cultivars lacked this gene. To clarify whether this was also true for wild chrysanthemum species, we performed Southern blot analysis. Genomic *CmCCD4a* has two *Bam*HI sites: one at −464 bp and the other at 1,565 bp from the start codon (Ohmiya et al. 2006). These sites are well conserved in *CmCCD4a* orthologs among wild chrysanthemum species (unpublished data). Then, the genomic DNA was digested with *Bam*HI

and probed with a digoxigenin-labeled *CmCCD4a* fragment. We detected signals that corresponded to *CmCCD4a* orthologs (approximately 2.0 kbp) in the lane of each white-flowered species, but no such band was observed in the lanes of yellow-flowered species (Fig. 1). This result was consistent with that obtained for chrysanthemum cultivars, suggesting that *CmCCD4a* orthologs are present in a wide range of chrysanthemum genera with white-colored ray petals.

The wild species that lacked ray petals, *C. shiwogiku* (lines 4-8 and 8808) and *C. pacificum* (lines 11 and 8706), were also analyzed. Surprisingly, *CmCCD4a* orthologs were detected in *C. shiwogiku* lines 4-8 and 8808 and *C. pacificum* line 11 (Fig. 1, lanes 11, 11′, and 12), but not in *C. pacificum* line 8706 (Fig. 1, lane 12′). Line 8706 thus appears to be a genetic variant of the original *C. pacificum* (which has *CmCCD4a* orthologs), because line 11 of the same species has the orthologs. This result demonstrated that *CmCCD4a* orthologs also exist in the apetalous wild species.

To confirm whether the proteins encoded by the *CmCCD4a* orthologs have enzymatic activity and can degrade carotenoids, interspecies crossing was performed between *C. shiwogiku* line 4-8 and the yellow-flowered cultivar 'Squash'. Line 8706 of *C. pacificum* was also crossed with 'Squash' to provide a negative control (Fig. 2a). When these two apetalous species were crossed with 'Squash', all progenies had ray petals. The same phenomenon was previously reported by Jong and Rademaker (1989) for interspecies crossings between chrysanthemum cultivars and *C. pacificum* 'IVT 78173'. Thus, the formation mechanism for ray petals appears to reflect the effects of dominant genes.

We then measured the color characteristics of the ray petals of the F_1 progenies obtained from the four-way crosses using a spectrophotometer, and plotted the a* and b* color values (Fig. 2b). The ray petals of the F_1 progenies divided completely into two separate clusters, with white or yellow petals, depending on whether *CmCCD4a* orthologs were present in the parents of the wild species. The ray petals of all F_1

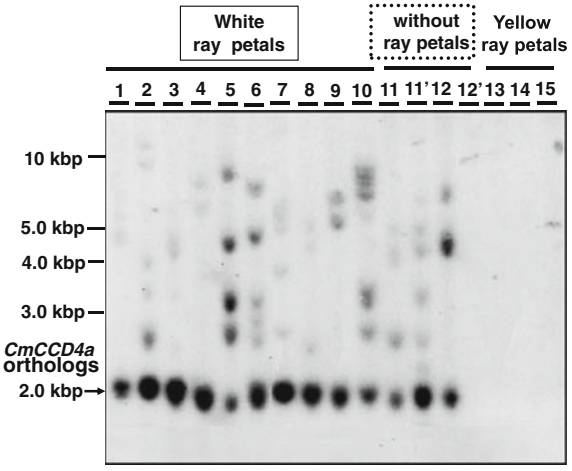

Fig. 1 The results of the Southern blot analysis for wild chrysanthemums (*Chrysanthemum* spp.). *Bam*HI-digested genomic DNA of each species (25 μg) was electrophoresed. Lanes 1–10, white-flowered wild species: 1, *C. wakasaense* line 2-12; 2, *C. ornatum* line 4-11; 3, *C. crassum* line 5-21; 4, *C. zawadskii* line 8912; 5, *C. weyrichii* line 8913; 6, *C. zawadskii* var. *latilobum* line 10; 7, *C. yoshinaganthum* line 12; 8, *C. japonense* line 13; 9, *C. makinoi* line 18; 10, *C. yezoense* line 19. Lanes 11–12′, species that lacked ray petals: 11 and 11′, *C. shiwogiku* lines 4-8 and 8808; 12 and 12′, *C. pacificum* lines 11 and 8706; lanes 13 and 14, yellow-flowered wild species: 13, *C. indicum* line 2-3; 14, *C. seticuspe* line 1. Lane 15, the yellow-flowered cultivar 'Squash'

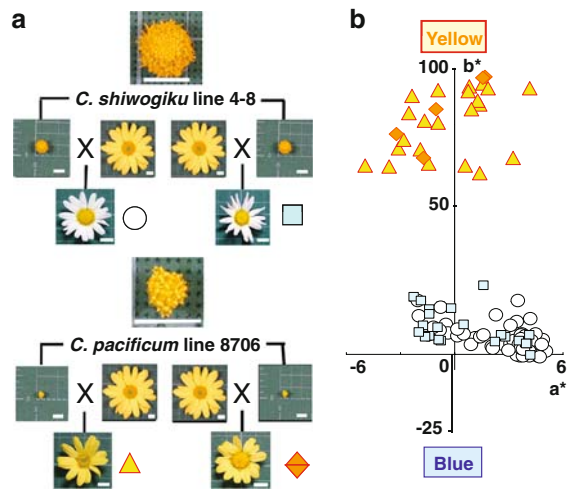

Fig. 2 **a** The combinations used in the interspecies crossing. The apetalous wild species *C. shiwogiku* line 4-8, which has *CmCCD4a* orthologs, and *C. pacificum* line 8706, which lacks these orthologs, were crossed with the yellow-flowered cultivar 'Squash' to obtain F_1 progenies. The white bars in each image represent 1 cm. **b** The scatterplot of the a* (x-axis) and b* (y-axis) color values for ray petals of the F_1 progenies. *Circles* and *triangles* indicate the results for the progenies from the crosses with *C. shiwogiku* line 4-8 and *C. pacificum* line 8706, respectively; the symbols correspond to that of the reciprocal crosses illustrated in **a**. The a* and b* values were defined as the color system by CIE in 1976

progenies with *C. shiwogiku* line 4-8 as the parent were white; this indicates that proteins encoded by *CmCCD4a* orthologs in *C. shiwogiku* line 4-8 exhibit enzymatic activity against carotenoids. In addition, the *CmCCD4a* orthologs of this line are homozygous. When *C. pacificum* line 8706, which lacks a *CmCCD4a* ortholog, was crossed with 'Squash', all progenies had yellow ray petals. Given the previous results (Jong and Rademaker 1989), in which the ray petals of F_1 progenies from *C. pacificum* 'IVT 78173' showed ray petals with various colors, depending on the crossing partner, *C. pacificum* 'IVT 78173' also appears to lack a *CmCCD4a* ortholog. Furthermore, all F_1 progenies obtained from the interspecies crossing between 'Squash' and all of the 10 white-flowered wild species in Fig. 1 had white ray petals (Hattori 1991: *C. japonense*; Sumitomo, unpublished data). The fact indicated that *CmCCD4a* orthologs played an important role in determining the ray petal color of these chrysanthemum genera. And enzymatic activities of translated proteins of *CmCCD4a* against carotenoids were confirmed not only by the results of previous RNAi experiments (Ohmiya et al. 2006) but also by our crossing experiments. We wanted to investigate whether the white coloration of ray petals in the progenies was associated with the presence of *CmCCD4a* orthologs. For this purpose, we used all the F_1 progenies (Fig. 2) and performed genomic PCR for the detection of specific genes. Figure 3 shows one representative progeny for each crossing combination. There was an evident relationship between the presence of *CmCCD4a* orthologs and the white coloration of ray petals. The bands that corresponded to *CmCCD4a* orthologs were observed in all white-flowered progenies but not in any yellow-flowered progenies. Thus, our results strongly suggested that proteins encoded by *CmCCD4a* orthologs are involved in the white coloration of petals of all examined chrysanthemums.

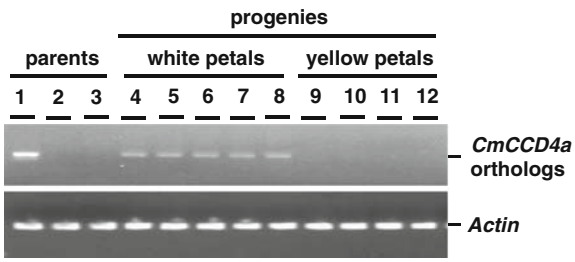

Fig. 3 The results of genomic PCR analysis of the parents used for the interspecies crossing and of their progenies. Lanes 1–3 represent the parent species: 1, *C. shiwogiku* line 4-8; 2, *C. pacificum* line 8706; 3, 'Squash' cultivar. Lanes 4–12 represent the progenies obtained by interspecific hybridization: 4–6, *C. shiwogiku* line 4-8 × 'Squash'; 7 and 8, 'Squash' × *C. shiwogiku* line 4-8; 9 and 10, *C. pacificum* line 8706 × 'Squash'; 11 and 12, 'Squash' × *C. pacificum* line 8706. Actin was used as the PCR control

CmCCD4a orthologs exist in a wide range of cultivars and wild species of chrysanthemums with white ray petals. Although cultivars and wild species are diverse, both chrysanthemum species groups were able to cross with little difficulty. Our results suggest that the chrysanthemum genera might have differentiated from a single ancestor that had one or more *CmCCD4a* orthologs. Wild species without ray petals might also have differentiated from species with white petals. Both white coloration and the formation of ray petals were shown to be dominant characteristics (Jong and Rademaker 1989; Hattori 1991). Thus, *C. pacificum* line 8706 may have lost the ability to produce ray petals as a result of evolutionary processes, and accidentally lost its *CmCCD4a* orthologs as a result of a mutation in a local population. Our results thus suggest that the translated proteins from *CmCCD4a* orthologs would maintain CCD function in chrysanthemums regardless of the existence of the ray petals.

To add useful characteristics from wild species to cultivars, *C. pacificum* and *C. shiwogiku* have been used for decades in interspecific crossbreeding (Shibata et al. 1988; Jong and Rademaker 1989; Tanikawa et al. 2006). The following beneficial characteristics have been reported: the development of several flower heads per leaf axil, significant stem elongation, prolific reproduction, frequent branching that avoids lodging, and resistance to disease and pest. Formerly, when these apetalous species were used as breeding parents, it was not possible to predict the ray petal color of the progenies before flowering. Our study revealed that the presence of *CmCCD4a* orthologs is useful because it allows the prediction of ray petal colors of the F_1 progenies before breeding.

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