Carotenoid Isomerase Is Key Determinant of Petal Color of Calendula officinalis*

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Background: Reddish 5-cis-carotenoids accumulate in the orange but not yellow petals of calendula.

Results: A CRTISO in orange petals of calendula lacks an isomerase activity.

Conclusion: CRTISO activity is a key factor in determining calendula petal color.

Significance: Cys-His-His at position 462 and Gly at position 450 of CoCRTISO are important for the isomerase activity.

Orange petals of calendula (Calendula officinalis) accumulate red carotenoids with the cis-configuration at the C-5 or C-5’ position (5-cis-carotenoids). We speculated that the orange-flowered calendula is a carotenoid isomerase (CRTISO) loss-of-function mutant that impairs the cis-to-trans conversion of 5-cis-carotenoids. We compared the sequences and enzyme activities of CRTISO from orange- and yellow-flowered calendulas. Four types of CRTISO were expressed in calendula petals. The deduced amino acid sequence of one of these genes (CoCRTISO1) was different between orange- and yellow-flowered calendulas, whereas the sequences of the other three CRTISOs were identical between these plants. Analysis of the enzymatic activities of the CoCRTISO homologs showed that CoCRTISO1-Y, which was expressed in yellow petals, converted carotenoids from the cis-to-trans-configuration, whereas both CoCRTISO1-ORa and 1-ORb, which were expressed in orange petals, showed no activity with any of the cis-carotenoids we tested. Moreover, the CoCRTISO1 genotypes of the F2 progeny obtained by crossing orange and yellow lines linked closely to petal color. These data indicate that CoCRTISO1 is a key regulator of the accumulation of 5-cis-carotenoids in calendula petals. Site-directed mutagenesis showed that the deletion of Cys-His-His at positions 462–464 in CoCRTISO1-ORa and a Gly-to-Glu amino acid substitution at position 450 in CoCRTISO1-ORb abolished enzyme activity completely, indicating that these amino acid residues are important for the enzymatic activity of CRTISO.

Carotenoids are one of the essential pigments in plants (1–4) and are generally responsible for the yellow to red color. The role of carotenoids in flowers is to attract insects that aid in pollination. Most carotenoid components in flowers are yellow and are generally responsible for the yellow to red color. The role of carotenoids in flowers is to attract insects that aid in pollination. Most carotenoid components in flowers are yellow.

xanthophylls, such as lutein, zeaxanthin, and violaxanthin, which furnish a pale to deep yellow color (5). They are synthesized and accumulate in chromoplasts in the esterified form (6–8). The petals of some plants have modified carotenoid biosynthetic pathways that produce carotenoids with specific compositions, associated with their respective genus or species, which express unique colors, such as orange and red, for example, astaxanthin in the dark red petals of Adonis aestivalis and Adonis annua (9, 10) and capsanthin in the red petals of the Asiatichybrid lily (11). Although the carotenoids that accumulate in petals are generally in the all-trans form, we previously demonstrated that the petals of some Asteraceae plants contain various types of cis-carotenoids, which are rarely found in plants; for example, the orange petals of calendula (Calendula officinalis), osteospermum (Osteospermum ecklonis), and Gazania spp. accumulate 5-cis-carotenoids2 (mostly 5-cis-lycopenes), which have the cis-configuration at the C-5 or C-5’ position (12, 13). These carotenoids are reddish and found in negligible quantities in yellow petals. Similarly, lycopene is rarely found in the petals of other plant species.

Carotenoids with various cis-configurations have been reported in plants; however, knowledge about their biosynthetic pathways is limited. The best known pathway is the introduction of the cis-configuration at C-7 (C-7’ and C-9 (C-9’)) into lycopene: during desaturation of phytoene to lycopene, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) simultaneously introduce the cis-configuration to the lycopene intermediates and produce (7Z,9Z,7’Z,9’Z)-lycopene (prolycopene) (14). Because the all-trans-configuration is required for subsequent lycopene cyclization, prolycopene is converted to the trans-configuration by carotenoid cis-trans-isomerase (CRTISO). CRTISO is a redox-type enzyme structurally related to the bacterial-type phytoene desaturase CRTI. A gene encoding CRTISO was first cloned from the cyanobacterium sl0033 gene (15, 16). Subsequently, CRTISOs were cloned from crtiso mutant plants such as arabidopsis (Arabidopsis thaliana) ccr2

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This article contains supplemental Tables S1–S13 and Figs. S1–S5.

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2 The abbreviations used are: 5-cis-carotenoid, carotenoid with a cis-configuration at the C-5 or C-5’ position; CRTISO, carotenoid isomerase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; prolycopene, (7Z,9Z,7’Z,9’Z)-lycopene; cTP, chloroplast/chromoplast transit peptides; 7,9-cis-carotenoid, carotenoid with cis-configuration at C-7 (C-7’) and C-9 (C-9’); RACE, rapid amplification of cDNA ends.
(17) and tomato (Solanum lycopersicum) mutant tangerine (18). These mutants accumulate prolycopene and other cis-isomers of its upstream precursors. Isaacson et al. (19) demonstrated that tomato CRTISO isomerizes adjacent cis-double bonds at C-7 and C-9 pairwise into the trans-configuration and functions in the carotenoid biosynthesis pathway by converting cis-lycopenes to all-trans-lycopene, a prerequisite for lycopene cyclization.

Accumulation of cis-carotenoids in the orange petals of calendula led us to speculate that the orange-flowered calendula is a "crtiso" loss-of-function mutant. Here, we cloned genes encoding CRTISO and compared their sequences in orange- and yellow-flowered calendulas. We found four types of CRTISO genes, one of which was expressed in a petal color-specific manner. We examined the enzymatic activity of the proteins encoded by the CRTISO homologs and showed that CRTISO is a key factor in the accumulation of 5-cis-carotenoids in calendula petals. We also provide useful information regarding a region of CRTISO that is important for its enzymatic activity.

EXPERIMENTAL PROCEDURES

Plant Materials—Four orange-flowered cultivars (Alice Orange, Pompon Orange, Orange Star, and Orange Gem) and four yellow-flowered cultivars (Alice Yellow, Pompon Yellow, Gold Star, and Golden Gem) of calendula (C. officinalis L.) were grown in greenhouses at the National Institute of Floricultural Science (Tsukuba, Ibaraki, Japan) (supplemental Fig. S1). HPLC Analysis of Carotenoids—Carotenoids were extracted from the petals of fully opened flowers and mature leaves and identified as described previously (12). Each extract was analyzed by means of HPLC (X-LC, JASCO, Tokyo, Japan) with a photodiode array detector (X-LC 3110MD, JASCO) under the following conditions: column, YMC carotenoid (250 × 4.6 mm inner diameter, 5 μm; YMC, Kyoto, Japan); solvent A, methanol/methyl tert-butyl ether/H₂O = 95:1:4 (v/v/v); solvent B, methanol/methyl tert-butyl ether/H₂O = 25:71:4; gradient, 0/100, 12/100, and 96/0 (min/% A); flow rate, 1.0 ml/min⁻¹; column temperature, 35 °C; and UV-visible monitoring range, 200–600 nm. To obtain substrates for the CRTISO enzyme assay, prolycopene and 5-cis-carotenoids were extracted from the fruits of a tangerine tomato mutant and from the orange petals of calendula, respectively. To remove xanthophylls, calendula extract was separated with equal volumes of hexane and aqueous 90% methanol, and the hexane epiphase was collected and evaporated. Individual cis-carotenoid species (prolycopene, (5Z,9Z)-lycopene, (5Z,9Z,5′Z)-lycopene, (5Z,9Z,5′Z,9′Z)-lycopene, (5′Z)-γ-carotene, and (5′Z)-rubixanthin) were isolated from the total carotenoid fraction using an HPLC system as described by Shimoto et al. (20). Because peaks of (5Z,9Z)- and (5Z,9Z,5′Z)-lycopene were very close and could not be separated, they were collected together.

Cloning of CRTISO cDNAs from Calendula Petals—Total RNA was isolated from the petals of fully opened flowers and mature leaves by using the cetyltrimethylammonium bromide method (21). cDNAs were synthesized by using the SuperScript first strand synthesis system (Invitrogen).

Partial length cDNAs encoding CRTISO and actin were amplified by means of RT-PCR with primers that corresponded to the highly conserved amino acid sequences among eudicot plants. Primer sequences for RT-PCR are shown in supplemental Table S1. cDNAs obtained from the petals of Alice Orange (orange flower) and Alice Yellow (yellow flower) served as PCR templates. Amplified PCR products of appropriate length were cloned into a pCR2.1 vector (TA cloning kit; Invitrogen) and sequenced with a Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

We amplified the 5′ cDNA ends of the gene from the petals of Alice Orange and Alice Yellow with the SMART RACE cDNA amplification kit (Clontech) according to the supplier’s protocols. Primers were designed from partial cDNA sequences using the Oligo computer software (Molecular Biology Insights, Cascade, CO). Then we designed 5′ end primers based on the 5′ RACE sequence and used them to amplify the full-length cDNAs encoding CRTISO. For full-length cDNA cloning, 3′ RACE cDNAs were used as templates. Primer sequences for 5′ RACE and the 5′ end of CRTISO are shown in supplemental Table S2. Four types of CRTISO homologs were identified using this cloning procedure (designated CoCRTISO1, CoCRTISO2, CoCRTISO3, and CoCRTISO4).

Phylogenetic Analysis and Prediction of the Presence of a Chloroplast/Chromoplast Transit Peptide—Multiple alignments of the deduced amino acid sequences of CRTISO were produced with a Web-based version of ClustalW. The phylogenetic tree was calculated by using the neighbor-joining method and bootstrap analysis (1000 replicates) using PHYLIP via the same Web site and was visualized with Treeview version 1.6.6. Prediction of the presence of chloroplast/chromoplast transit peptides (cTP) in the protein sequences and the location of potential cTP cleavage sites were carried out using the ChloroP 1.1 server (22).

Quantitative Real Time PCR Analysis—We performed real time quantitative PCR to quantify the mRNAs of the CoCRTISO homologs in petals and leaves. Total RNAs were isolated from the petals of fully opened flowers and mature leaves by means of the cetyltrimethylammonium bromide method (21). cDNAs were synthesized by using the SuperScript first strand synthesis system (Invitrogen) from total RNA treated with DNase I. Transcript levels were analyzed with the SYBR Premix Ex Taq kit (TaKaRa, Shiga, Japan) and a Thermal Cycler Dice real time system (TaKaRa) according to the manufacturer’s instructions. Primers specific to each CRTISO homolog for PCR were designed using the Oligo software based on the full-length cDNA sequences, avoiding homologous regions among the CoCRTISO homologs, and were checked for specific product formation by using PCR. Actin primers were also designed from the partial cDNA sequence. Primer sequences are shown in supplemental Table S3. The concentration of actin mRNA in each sample was determined to normalize the values and account for differences in the amount of total RNA.

Site-directed Mutagenesis of CRTISO and Construction of Expression Vectors—The coding regions of CoCRTISO homologs were amplified by using PCR with 5′ end primers with the BstZ17I restriction site, 3′ end primers with the Sall
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restriction site, and Prime STAR HS DNA polymerase (Takara). Primer sequences are shown in supplemental Table S4. The open reading frame of each gene was shortened by deleting the predicted transit peptide sequence. The fragments were cloned into a pBlunt-TOPOII vector (Invitrogen) and digested with BstZ17I and SalI. Then the fragments were subcloned into a pMAL-c2x vector (New England Biolabs, Ipswich, MA) digested with XmnI and SalI. The recombinant proteins contained a maltose-binding protein at the C terminus.

Additionally, we made a CoCRTISO1 site-directed mutant series, named m1 to m11 (see Table 1 and Fig. 1). The mutations were introduced by use of overlap extension PCR (23): fragments containing 20–50-bp overlap regions were amplified by using PCR (first PCR) with plasmid DNAs as templates and with mutagenic primers. The fragments were mixed and subjected to PCR amplification by using a 5’ end primer with a BstZ17I restriction site and a 3’ end primer with a SalI restriction site for CoCRTISO1 (second PCR). Primer sequences and the template and primer combinations for the first PCR are shown in supplemental Tables S5 and S6. The amplified products were subcloned into a pBlunt-TOPOII vector and digested with BstZ17I and SalI. The fragments were then inserted into a pMAL-c2x vector digested with XmnI and SalI.

**Expression and Extraction of CoCRTISO in Escherichia coli—** E. coli cells of the strain BL21 carrying the pMAL-CoCRTISO plasmids were grown in LB medium containing ampicillin (100 mg liter\(^{-1}\)) at 37 °C until absorbance at 550 nm reached 0.5. Isopropyl thio-\(\beta\)-\(D\)-galactoside was then added (0.5 mM) for 12–15 h at 25 °C to induce the expression of the recombinant genes. Bacterial cells were harvested, resuspended in one-tenth volume of cold incubation buffer (1% Triton-X, 100 mM Tris, 10 mM MgCl\(_2\), pH 7.4), and ruptured by using an ultrasonic cell disruptor at 10–15 W m\(^{-2}\) (Microson XL; Misonix, Farmingdale, NY). The lysate was centrifuged at 13,000 \(\times g\) for 15 min at 4 °C. The clear supernatant (soluble fraction) was collected and kept on ice, either for the in vitro assay or for protein purification. Affinity chromatography of recombinant proteins was carried out by use of an amylose resin column (New England Biolabs) according to the manufacturer’s instructions.

**In Vitro Enzyme Assay—** An in vitro enzyme assay was carried out basically as described by Isaacson et al. (19). Carotenoids (~1 mg ml\(^{-1}\)) were dissolved in incubation buffer by sonication. To test the activity of CoCRTISO and its mutant enzymes, we incubated 500 \(\mu\)l of crude enzyme extracts containing ~20 \(\mu\)g of substrate at 30 °C in the dark for 3 h. To test the enzymatic activity and substrate specificity of CoCRTISO1, -2, -3, and -4, we incubated 200 \(\mu\)g of purified enzymes, 25 mg of catalase, 150 mg of glucose oxidase, 750 mg of glucose, and ~5 \(\mu\)g of substrate in a total volume of 500 \(\mu\)l of incubation buffer at 30 °C in the dark for 3 h. After the reaction, 200 \(\mu\)l of acetone and 200 \(\mu\)l of diethyl ether were added to the reaction mixture, which was then centrifuged at 10,000 \(\times g\) for 5 min. The organic phase was collected, dried in vacuo, dissolved in 100 \(\mu\)l of methanol, and subjected to HPLC analysis.

**Western Blot Analysis—** Protein samples were loaded onto a 12.5% SDS-PAGE gel (ATTO, Tokyo, Japan) and blotted onto nitrocellulose membrane (Hybond C’; GE Healthcare, Buckinghamshire, UK). The membranes were blocked with 2% BSA in Tris-buffered saline, pH 7.4, and incubated with anti-maltose-binding protein antiserum (New England Biolabs) at a 1:1000 dilution and then with secondary alkaline phosphatase-conjugated goat-anti rabbit IgG antibodies (Bio-Rad) at 1:300 dilution. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used for chromogenic detection.

**Reciprocal Crossing between Orange- and Yellow-flowered Calendula—** To investigate the inheritance of flower color, we crossed orange- and yellow-flowered calendulas for two generations. Calendula is allogamous, so no seeds are set by self-pollination. Therefore, we crossed the cultivars as follows: first, we created eight lines by crossing orange-flowered with orange-flowered cultivars (Alice Orange × Orange Star, Alice Orange × Orange Gem, and Orange Star × Alice Orange), yellow-flowered with orange-flowered cultivars (Alice Yellow × Alice Orange, Gold Star × Orange Star, and Alice Orange × Alice Yellow), and yellow-flowered with yellow-flowered cultivars (Golden Gem × Alice Yellow and Alice Yellow × Golden Gem) and analyzed the flower color of the progeny (F\(_1\) generation). Next, we created an F\(_2\) generation to analyze the segregation of flower color (supplemental Fig. S2). It was difficult to obtain sufficient quantities of F\(_2\) seeds from the crosses between the above-mentioned F\(_1\) progenies because of inbreeding depression; therefore, we used two lines, the orange-flowered progeny of Alice Orange × Orange Star and the yellow-flowered progeny of Alice Yellow × Golden Gem, as parents for the crossing test. The progeny of these lines were crossed with each other, and the seeds obtained were analyzed as the F\(_2\) generation.

**Isolation of Genomic DNA and Genomic PCR to Identify the Genotype of CoCRTISO1—** The genomic DNA of F\(_2\) plants was isolated from immature leaves by using Nucleon PhytoPure (GE Healthcare) according to the manufacturer’s instructions. To identify the genotypes of the F\(_2\) plants and the calendula cultivars, we performed genomic PCR. Primer sequences specific to each CoCRTISO1 homolog are shown in supplemental Table S7. PCR amplification was carried out in a TP-3000 thermal cycler (Takara) with Z-Taq DNA polymerase (Takara) according to the manufacturer’s instructions.

**RESULTS**

**Cloning of CRTISO Genes from Calendula Petals—** We performed RT-PCR-mediated cloning of CRTISO genes by using primers that corresponded to the highly conserved region among eudicot plants and isolated four CRTISO homologs from an orange-flowered Alice Orange cultivar and from a yellow-flowered Alice Yellow cultivar (supplemental Fig. S3). The full-length sequence of one of these homologs (designated CoCRTISO1) differed between Alice Orange and Alice Yellow. The sequences of the other homologs (designated CoCRTISO2, -3, and -4) were identical between the two cultivars. In addition, we isolated full-length CoCRTISO1 cDNA clones from orange- and yellow-flowered cultivars. All of the CoCRTISO1 sequences from the yellow-flowered cultivars were identical (designated CoCRTISO1-Y). In contrast, the CoCRTISO sequences from the orange-flowered cultivars were of two types (designated CoCRTISO1-ORA and CoCRTISO1-ORB). CoCRTISO1-ORA was isolated from Alice Orange and Orange Star; CoCRTISO1-
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1. **Expression of CoCRTISO in Petals and Leaves**—We designed primer sets specific to each CoCRTISO homolog for real time quantitative PCR analysis and compared the expression levels of these homologs between orange- and yellow-flowered cultivars. We found that CoCRTISO1-Y was expressed only in the petals and leaves of yellow-flowered cultivars and that CoCRTISO1-OR was expressed only in the petals and leaves of orange-flowered cultivars (Fig. 2). On the other hand, CoCRTISO2, -3, and -4 were expressed in the petals of both orange- and yellow-flowered cultivars. The expression levels of CoCRTISO2, -3, and -4 were very low compared with those of CoCRTISO1; they were ~1/150 to 1/2000 of those of CoCRTISO1-Y. All homologs, except CoCRTISO2, were expressed at higher levels in petals than in leaves.

2. **In Vitro Enzymatic Activity of CoCRTISO1 and Its Mutant Polypeptides**—We examined the enzymatic activity of CoCRTISO1-Y, -1-ORa, and -1-ORb expressed in *E. coli*. *E. coli* cells carrying pMAL-CoCRTISO1-Y, -1-ORa, or -1-ORb accumulated a polypeptide of an apparent molecular mass of ~100 kDa, matching the predicted size of the mature CoCRTISO1 polypeptide (58 kDa) plus the maltose-binding protein (42 kDa) (supplemental Fig. S4). Crude enzyme extracts (soluble fraction of an *E. coli* lysate) were used to assay the enzymatic activity of CoCRTISO1 in vitro. Equivalent extracts of *E. coli* cells transformed with the empty vector pMAL-c2x served as controls. Carotenoids extracted from tangerine tomato fruits (which contain mainly prolycopene) and from the orange petals of calendula (which contain 5-cis-carotenoids) served as substrates. Compared with the control, CoCRTISO1-Y caused an increase in the percentage of (all-E)-lycopene in the extracts from both tangerine tomato and calendula, indicating that CoCRTISO1-Y functioned as an isomerase for the cis-to-trans conversion (Fig. 3, Table 1, and supplemental Table S8). On the other hand, reaction mixtures of CoCRTISO1-ORa and -1-ORb produced the same HPLC chromatograms as that of the control, indicating that these proteins had no isomerase activity.

To determine the amino acid residue or residues that were important for the isomerase activity, four divergent amino acid residues and a three-amino acid sequence, which were con-
served among higher plants, were chosen for site-directed mutagenesis (Fig. 1). We constructed 11 CoCRTISO1 mutants (m1 to m11) such that the divergent amino acid residues of CoCRTISO1-ORa or 1-ORb were introduced into 1-Y or vice versa, and those fragments were then inserted into an expression vector, pMAL-c2x. E. coli cells carrying pMAL-Co-CRTISO1 to m11 accumulated polypeptides of an apparent molecular mass of ~100 kDa, matching the predicted size (data not shown). Among the constructs bearing the divergent amino acid residues of CoCRTISO1-Y that were introduced into CoCRTISO1-ORa (m2, m7, and m9) or CoCRTISO1-ORb (m3, m5, and m11), m7 (1-ORa462insCHH) and m5 (1-ORbE450G) restored the activity, whereas m2 (1-ORaI370T), m3 (1-ORbI370T), m9 (1-ORaL581P), and m11 (1-ORbS412A) lacked enzyme activity (Fig. 3 and Table 1). On the other hand, among the constructs bearing the divergent amino acid residues of CoCRTISO1-ORa or -1-ORb that were introduced into CoCRTISO1-Y (m1, m4, m6, and m10), isomerase activity was retained in m1 (1-YT370I) and m10 (1-YA412S) but was abolished in m4 (1-YG450E) and m6 (1-YCHH462del).

In Vitro Enzymatic Activity and Substrate Specificity of CoCRTISO—We analyzed differences in the enzyme activity and substrate specificity of the CoCRTISO homologs. Enzymes were purified with an amylose resin from crude cell extracts of E. coli carrying pMAL-CoCRTISO1, -2, -3, and -4. Western blot analysis showed that the purified fractions contained the desirable polypeptides of an apparent molecular mass of ~100 kDa (supplemental Fig. S4).

In vitro conversion from cis- to trans-configuration was examined with the purified proteins (Fig. 4, Table 1, and supplemental Table S9). The conversion rate from the 7,9,7',9'-cis-configuration of prolycopene to the all-trans-configuration was relatively low in CoCRTISO3 and -4 compared with that in CoCRTISO1 and -2. On the other hand, in vitro conversion from the 5-cis- to the trans-configuration was dependent on the carotenoid species (Fig. 4, Table 1, and supplemental Fig. S5 and Tables S10–S13). When (5Z,9Z,5',9'-Z)-lycopene, (5'Z)-γ-carotene, and (5'Z)-rubixanthin were used as substrates, the proportions of (all-E)-lycopene, (all-E)-γ-carotene, and (all-E)-rubixanthin, respectively, were increased by the catalytic activ-

FIGURE 2. Expression of CoCRTISO homologs in orange- and yellow-flowered cultivars. Quantitative real time RT-PCR analysis was performed in triplicate by using primers specific to each homolog; expression levels were normalized against actin levels. The mean values ± S.E. are shown. AO, Alice Orange; OS, Orange Star; PO, Pompom Orange; OG, Orange Gem; AY, Alice Yellow; GS, Gold Star; PY, Pompom Yellow; GG, Golden Gem.

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[Graphs showing expression levels of CoCRTISO homologs in different cultivars.]
ities of CoCRTISO1-Y and -2. However, these conversions of the reactant were not detected with CoCRTISO3 and -4. In the case of (5Z,9Z)- and (5Z,9Z,5Z)-lycopene, conversion to (all-E)-lycopene was detected with CoCRTISO1-Y, -2, -3, and -4. CoCRTISO1-OR lacked enzymatic activity when prolycopene and 5-cis-carotenoids served as substrates.

Inheritance of Petal Color and the Relationship between CoCRTISO1 Genotype and Petal Color

To determine the inheritance of petal color of calendula, we performed crossing experiments. All of the F1 progenies obtained from crosses between yellow- and yellow-flowered cultivars and between yellow- and orange-flowered cultivars had yellow petals, and all of the F1 progenies obtained from crosses between orange- and orange-flowered cultivars had orange petals. The yellow-flowered progenies in the crosses between yellow- and orange-flowered lines were crossed with each other, and the petal color of the F2 progenies was analyzed (supplemental Fig. S2). The 146 F2 progenies segregated for 102 yellow-flowered and 44 orange-flowered individuals. These results indicate that the yellow petal color was dominant over orange and was a monogenic character (Table 2).

Genomic PCR with primers specific to either CoCRTISO1-Y or -1-OR was performed to examine the genotypes of CoCRTISO in the F2 progenies. All of the orange-flowered F2 progenies showed DNA amplification only with primers specific to CoCRTISO1-OR (Table 2 and Fig. 5). On the other hand,
of the 102 yellow-flowered F2 progenies, 69 showed DNA amplification with primers specific to both CoCRTISO1-Y and -1-OR, and 33 progenies showed DNA amplification only with primers specific to CoCRTISO1-Y. Additionally, in the yellow-flowered cultivars tested, amplification was detected only with primers specific to CoCRTISO1-Y; in the orange-flowered cultivars tested, amplification was detected only with primers specific to CoCRTISO1-OR. These results indicate that the orange-flowered progenies had a homozygous genotype of CoCRTISO1-OR and that the yellow-flowered progenies had a homozygous genotype of CoCRTISO1-Y or a heterozygous genotype of CoCRTISO1-Y and -1-OR. All of the orange- and yellow-flowered cultivars tested had homozygous genotypes of CoCRTISO1-OR and CoCRTISO1-Y, respectively.

**DISCUSSION**

Our previous work demonstrated that the orange petal color of calendula is due to the accumulation of reddish cis-carotenoids, mainly 5-cis-lycopenes (12). Based on the assumption that a difference in CRTISO enzyme activity between yellow- and orange-flowered calendula was responsible for the difference in petal color, we analyzed nucleotide sequences and enzymatic activities of CoCRTISO homologs between yellow- and orange-flowered calendula. Recently, CRTISO sequences from various plant species have been deposited in DNA databases. However, no plant in the databases appears to have multiple types of CRTISO genes, except maize (24). Here, we showed that calendula CRTISO forms a small multigene family of at least four CRTISO homologs (CoCRTISO1, -2, -3, and -4) that were expressed in petals (supplemental Fig. S3). Deduced amino acid sequences of CoCRTISO2, -3, and -4 were completely identical between orange- and yellow-flowered calendulas, whereas there were several differences between CoCRTISO1-Y from yellow-flowered calendula and
CoCRTISO1-ORa and -1-ORb from orange-flowered calendula. Real time quantitative PCR analysis showed that CoCRTISO1-Y was expressed only in yellow-flowered cultivars and that CoCRTISO1-OR (total of -1-ORa and -1-ORb) was expressed only in orange-flowered cultivars. These results suggest that CoCRTISO1 is a key determinant of the petal color of calendula.

The results of an in vitro enzyme assay (Table 1, Fig. 4, and supplemental Tables S9–S13 and Fig. S5) showed that CoCRTISO1-Y can convert carotenoids from the cis-to-trans-configuration, whereas CoCRTISO1-ORa and -1-ORb lacked activity with any of the cis-carotenoids tested. CoCRTISO1-Y showed wide substrate specificity for cis-carotenoids, converting both 7,9 (7’,9’)- and 5,9 (5’,9’)-cis-configurations to the trans-configuration. CoCRTISO-Y converted prolycopene, (5Z,9Z,5’Z,9’Z)-lycopene, (5Z,9Z,5’Z,9’Z)-lycopene, and (5Z,9Z)-lycopene, which are composed of only polyene chains (ψ-end), and (5’Z)-γ-carotene and (5’Z)-rubixanthin, which have a β-ring configuration on one side. CoCRTISO2, -3, and -4 had less isomerase activity with prolycopene than did CoCRTISO1-Y, and activities with 5-cis-carotenoids were very low or undetectable.

CoCRTISO2, -3, and -4 were expressed in all cultivars regardless of petal color; however, the expression levels of these homologs in petals were extremely low compared with that of CoCRTISO1: 1/150 to 1/2000 of CoCRTISO1-Y. These results indicate that, in yellow petals, most of the CRTISO activity is derived from CoCRTISO1-Y, and most 5-cis-carotenoids are
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converted to trans-carotenoids. By contrast, in orange petals, the total CRTISO activity is low because of the lack of the isomerase activity of both CoCRTISO1-OrA and -1-OrB, which may result in the accumulation of 5-cis-carotenoids.

To test the hypothesis, we crossed orange- and yellow-flowered cultivars (supplemental Fig. S2). All of the F1 progenies obtained by reciprocal crosses between orange- and yellow-flowered cultivars had yellow petals, indicating that yellow petal color is dominant over orange. Moreover, the F2 progenies showed a ~3:1 (yellow:orange) segregation ratio (Table 2). In addition, genomic PCR analysis of F2 progenies with primers specific to either CoCRTISO1-Y or -1-Or (Fig. 5) showed that all of the CoCRTISO1 genotypes were closely linked to the petal color of the F2 progenies: orange-flowered progenies had a homozygous genotype of CoCRTISO1-OrA, and yellow-flowered progenies had either a homozygous genotype of CoCRTISO1-OrB, or a heterozygous genotype of CoCRTISO1-Y and -1-Or. These results support the hypothesis that CoCRTISO1-Y and -1-Or determine the petal color of calendula.

To clarify the cause of the loss-of-function of CoCRTISO1-OrA and -1-OrB, we performed site-directed mutagenesis of the CoCRTISO1 homologs. The polypeptide of CoCRTISO1-OrA includes four amino acid substitutions and the deletion of a three-amino acid sequence, whereas that of CoCRTISO1-OrB includes four amino acid substitutions compared with CoCRTISO1-Y (Fig. 1). We then constructed a series of 11 site-directed mutant genes and tested the enzymatic activities of their translation products to determine which divergent amino acids cause the inactivation of CoCRTISO1-OrA and -1-OrB (Table 1). We found that deletion of Cys-His-His at positions 462–464 in CoCRTISO1-OrA and a Gly-to-Glu substitution at position 450 in CoCRTISO1-OrB completely abolished the enzyme activity. These amino acid residues are highly conserved from cyanobacteria to higher plants, suggesting that they are essential for CRTISO enzyme activity. This is the first report demonstrating the importance of those amino acid residues for CRTISO activity.

How 5-cis-carotenoids in calendula petals are biosynthesized remains unknown. Generally, carotenoids with the 5-cis-configuration are very rare in plants, and besides calendula, only a few examples have been reported, such as (5′Z)-rubixanthin in the orange petals of gazania (13, 25), (5Z,9Z,5′Z)-lycopene and (5Z)-γ-carotene in the orange petals of osteospermum (13), (5Z)-neurosporene and (5′Z,13Z)- or (5′Z,13′Z)-rubixanthin in rose hips (26), and (3′Z,5′Z)-celaxanthin and (3′Z,5′Z)-tornulin in the seeds of Celastrus orbiculatus (27). In the orange petals of calendula, 10 kinds of 5-cis-carotenoids have been detected (12); however, carotenoids with the cis-configuration at C-7 (C-7′) and C-9 (C-9′) (7,9-cis-carotenoids), including prolycopene, which is a typical substrate of CRTISO in carotenoid biosynthesis, were not detected. All crtiso mutants reported in cyanobacteria (15), tomato (18), arabidopsis (17), and rice (28) accumulate prolycopene. In addition, there is increasing evidence that, in plants, PDS and ZDS produce 7,9-cis-carotenoids during desaturation of phytoene and 5-carotene, respectively (14, 29, 30, 31), suggesting that the pathway from phytoene to lycopene via 7,9-cis-carotenoids is a universal pathway across the plant kingdom. Here, we propose two hypotheses to explain why the orange petals of calendula do not accumulate 7,9-cis-carotenoids but accumulate 5-cis-carotenoids: first, both 7,9- and 5-cis-carotenoids are produced during lycopene biosynthesis, but 7,9-cis-carotenoids are preferentially isomerized by CoCRTISO2, -3, and -4; consequently, 7,9-cis-carotenoids are not detected in orange-flowered calendula. The second hypothesis is that 7,9-cis-carotenoids are not synthesized at all, and lycopene is biosynthesized via 5-cis-carotenoids in calendula (Fig. 6). In either case, the PDS and ZDS of calendula would be able to produce 5-cis-carotenoids as intermediates of all-trans-lycopene biosynthesis.

In conclusion, we propose that the mechanism regulating the amount of 5-cis-carotenoids in calendula petals is as follows: in yellow petals, 5-cis-carotenoids, which are produced during the course of carotenoid biosynthesis, are changed into trans-carotenoids mainly by CoCRTISO1-Y. The trans form ends of the polyene chain are immediately cyclized and hydroxylated, and finally these carotenoids accumulate as yellow xanthophylls. On the other hand, the orange petals have low CRTISO activity because CoCRTISO1-OrA, a major CoCRTISO homolog expressed in these petals, is inactive. Total CRTISO activity, which is derived from CoCRTISO2, -3, and -4, is insufficient for the cis-to-trans conversion of the substantial amount of 5-cis-carotenoids in orange petals; therefore, only a fraction of the 5-cis-carotenoids is converted to the trans form, leaving the rest of the 5-cis-carotenoids to accumulate. The results obtained in this study demonstrate that the conversion of lycopene from the cis- to the trans-configuration is an important process for lycopene cyclization. The question of how 5-cis-carotenoids are synthesized remains. Further research is needed on the function of PDS and ZDS to fully understand carotenoid biosynthesis in the petals of calendula.

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