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ORIGINAL PAPER

Alteration of flower colour in *Ipomoea nil* through CRISPR/Cas9-mediated mutagenesis of *carotenoid cleavage dioxygenase 4*

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Abstract Japanese morning glory, *Ipomoea nil*, exhibits a variety of flower colours, except yellow, reflecting the accumulation of only trace amounts of carotenoids in the petals. In a previous study, we attributed this effect to the low expression levels of carotenogenic genes in the petals, but there may be other contributing factors. In the present study, we investigated the possible involvement of carotenoid cleavage dioxygenase (CCD), which cleaves specific double bonds of the polyene chains of carotenoids, in the regulation of carotenoid accumulation in the petals of *I. nil*. Using bioinformatics analysis, seven *InCCD* genes were identified in the *I. nil* genome. Sequencing and expression analyses indicated potential involvement of *InCCD4* in carotenoid degradation in the petals. Successful knockout of *InCCD4* using the CRISPR/Cas9 system in the white-flowered cultivar *I. nil* cv. AK77 caused the white petals to turn pale yellow. The total amount of carotenoids in the petals of *ccd4* plants was increased 20-fold relative to non-transgenic plants. This result indicates that in the petals of *I. nil*, not only low carotenogenic gene expression but also carotenoid degradation leads to extremely low levels of carotenoids.

Keywords Carotenoid cleavage dioxygenase · Carotenoid metabolic engineering · CRISPR/Cas9 · Flower colour alteration · *Ipomoea nil* · Targeted mutagenesis

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Introduction

The Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*) is one of the two traditional horticultural model plants selected for the National BioResource Project by the Agency for Medical Research and Development (AMED) in Japan (Yamazaki et al. 2009). Since the seventeenth century, reflecting transposon mutagenesis in the anthocyanin biosynthetic pathway, the flowers of *I. nil* have come to display several different colours (e.g., red, pink, purple, brown and white), while the original cultivar has pale blue petals. However, bright yellow-flowered cultivars of *I. nil* do not exist. Although some cultivars contain small amounts of yellow pigments, such as aurones (Saito et al. 1994), these acyanic flowers are only pale yellow in colour. Carotenoids are another yellow pigment responsible for the colouration of flower petals, but *I. nil* accumulates only trace amounts of carotenoids in its petals (Yamamizo et al. 2010).

Several strategies for controlling carotenoid accumulation in floral tissues have been reported, involving the synthesis, sink capacity, esterification, and suppression of the degradation of carotenoids (reviews: (Tanaka and Ohmiya 2008; Li and Yuan 2013)). For example, in marigold, *Tagetes erecta*, different levels of carotenoid accumulation lead to differences in petal colour, ranging from pale yellow to dark orange (Moehs et al. 2001). A previous study showed that the carotenoid content correlates well with the transcript levels of carotenogenic genes such as *phytoene synthase* (*PSY*) and *1-deoxy-d-xylulose 5-phosphate synthase* (Moehs et al. 2001). This finding suggests that the carotenoid content depends on the mRNA transcript levels or stability of carotenogenic genes in tissues (Moehs et al. 2001). Previously, we reported that the transcription levels of carotenogenic genes such as *PSY* and *β-ring hydroxylase* (*CHYB*) in the petals of *I. nil* were extremely low compared with those of *I. obscura* var. *lutea* (formally *Ipomoea* sp.), which exhibits high levels of carotenoids and bears vivid yellow petals (Yamamizo et al. 2010). Subsequently, carotenoid biosynthetic pathway in the floral tissue of *I. nil* was enhanced by introducing multiple carotenogenic genes of which expression levels are relatively low in the petals of *I. nil* than that of *I. obscura* var. *lutea*; however, the petal colour did not change (Watanabe et al. in press). We concluded that the trace amounts of carotenoids in the petals of *I. nil* were not due solely to low expression levels of carotenogenic genes but that another mechanism must prevent carotenoid accumulation.

In higher plants, carotenoid cleavage dioxygenase (CCD) enzymes contribute to carotenoid degradation and cleave specific double bonds of the polyene chain of carotenoids. CCD was first identified through the analysis of a viviparous abscisic acid-deficient mutant of maize (*Zea mays*) and was designated *viviparous14* (*VP14*) (Schwartz et al. 1997; Tan et al. 1997). Based on studies focussing on *VP14*, many CCD homologous enzymes in different plant species and other organisms have been identified and characterized [reviews: (Ohmiya 2009; Walter et al. 2010; Ahrazem et al. 2016)]. Analysis of the genome sequence of *Arabidopsis thaliana* led to the definition of nine clades of dioxygenases (Tan et al. 2003), which are classified into the *9-cis-epoxycarotenoid dioxygenase* (*NCED*) and *CCD* subfamilies based on sequence similarities. NCEDs are related to the production of viviparous abscisic acid but not to petal colour formation (Schwartz et al. 1997; Tan et al. 1997; Schwartz et al. 2003). However, the functions of CCDs differ between clades (Ahrazem et al. 2016). CCD7 and CCD8 are related to the production of strigolactones, which act as a branching signal (Gomez-Roldan et al. 2008; Umehara et al. 2008). CCD1 plays roles in the formation of volatile compounds responsible for flavour and aroma and in carotenoid turnover (Schwartz et al. 2001; Auldridge et al. 2006; Ilg et al. 2010). The functions of CCD4 are similar to those of CCD1, but some CCD4s are involved in the degradation of carotenoids and prevent carotenoid accumulation in the petals. In chrysanthemum (*Chrysanthemum morifolium*), the expression levels of carotenogenic genes show no significant difference between white and yellow petals. However, *CmCCD4a* is expressed in white petals but not in yellow petals (Kishimoto and Ohmiya 2006; Ohmiya et al. 2006). Furthermore, loss-of-function mutation of CmCCD4a in white chrysanthemum petals resulted in a drastic increase in carotenoids (Ohmiya et al. 2012). Similarly, transposon mutagenesis of *BnaC3.CCD4*, an orthologue of *CCD4* in *Brassica napus*, converts petal colour from white to yellow (Zhang et al. 2015). These results indicate that CCD4 cleaves carotenoids and prevents carotenoid accumulation in the white petals of chrysanthemum and *B. napus*. However, although *CCD4*s are expressed in the petals of rose

(Huang et al. 2009), tomato (Wei et al. 2016) and Arabidopsis (Winter et al. 2007) (eFP browser; http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi), the involvement of CCD4 in petal colour formation has not been clearly elucidated in these plants. In *I. nil*, a substantial amount of InCCD4 is expressed in the petals (Yamamizo et al. 2010). However, in a previous study, only the petal-specific expression of InCCD4 was examined based on a partial sequence (Yamamizo et al. 2010); thus, the contribution of InCCD4 remains unknown, and a comprehensive study of InCCDs is needed.

Previous attempts to change flower colour by suppressing *CCD4* expression using RNAi methods have been reported (Ohmiya et al. 2006, 2009). In *C. morifolium*, the introduction of a *CmCCD4a* RNAi construct into a white-flowered cultivar successfully reduced the level of *CmCCD4a* expression in the petals. The carotenoid content in the petals of the RNAi transformants increased to 102 μg g^{-1} but was still much lower than in yellow-flowered cultivars because *CmCCD4a* expression remained at low levels (Kishimoto et al. 2007; Ohmiya et al. 2009). Thus, completely knocking out *CCD4* expression might be necessary to obtain a deeper yellow petal colour. Recently developed methods for targeted mutagenesis (or genome editing) can completely knock out gene function. In particular, the clustered regularly interspaced short palindromic repeat-associated endonuclease 9 (CRISPER/Cas9) system can be utilized more easily than other systems because of its simple experimental design and high specificity (Puchta 2017), and the CRISPR/Cas9 system has already been successfully applied in *I. nil* (Watanabe et al. 2017).

In the present study, we comprehensively analysed all the predicted *CCD* homologous genes according to the whole-genome sequence of *I. nil* (Hoshino et al. 2016). The sequencing and expression analyses suggested potential involvement of *InCCD4* in carotenoid degradation in the petals. Therefore, we knocked out *InCCD4* using targeted mutagenesis with CRISPR/Cas9 system. The *Inccd4* knockout plants exhibited pale yellow petals with up to 20-fold-increased carotenoid accumulation in the midribs of the petal. Subsequently, we revealed that *InCCD4* is also related to petal colour formation in *I. nil*, similar to what has been found in chrysanthemum and Brassica.

Materials and methods

Plant materials and growth conditions

Seeds of *I. nil* cv. AK77 (obtained from the National BioResource Project (NBRP) "Morning glory") were used in the experiments. The seedlings were grown in vermiculite fertilized with a 1000-fold-diluted Hyponex 6-10-5 solution (Hyponex Japan, Tokyo, Japan) once a week under long-day conditions (16 h light: 8 h dark; 60 μmol m^{-2} s^{-1}, FL40SW lamps; NEC Lighting Ltd., Tokyo, Japan) at 25 °C for 2 weeks. The plants were then transferred to short-day conditions (8 h light: 16 h dark) at 25 °C. For RT-qPCR, roots, stems and cotyledons were collected at 1 week after sowing. Leaves were collected at 2 weeks after sowing. Petals were collected at each developmental stage, 96, 48, and 12 h before flowering, and immediately after opening (referred to as S1, S2, S3 and S4, respectively). For transformation, immature embryos of *I. nil* cv. AK77 were collected at 2 weeks after flower opening (Ono et al. 2000).

Identification of *CCD* genes in plants

A tBLASTn (DNA Data Bank of Japan, Shizuoka, Japan) (Altschul et al. 1997) search was performed using the amino acid sequences of the Arabidopsis *CCD* genes as the query against the predicted transcript sequence from the *I. nil* genome and the EST sequence database (Hoshino et al. 2016), using an e-value of 1e-20. *InCCD4* was also detected in the EST database (http://ipomoeanil.nibb.ac.jp/; Contig11866d).

Phylogeny analysis

Multiple alignments of amino acid sequences were produced using GENETYX-MAC (Software Kaihatsu Co., Tokyo, Japan). The phylogenetic tree was calculated using the neighbour-joining method (Saitou and Nei 1987) and bootstrap analysis (1000 replicates) with MEGA7 software (Kumar et al. 2016). Missing sequence data were treated via pairwise deletion of gaps. Branch lengths were assigned utilizing pairwise calculations of genetic distances.

Quantitative real-time PCR (RT-qPCR) analysis

Total RNA was isolated from each tissue and petals from each stage using the Get Pure RNA Kit (Dojindo, Kumamoto, Japan). Subsequently, cDNA was synthesized from total RNA (1.0 μg) using the SuperScriptIII First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and oligo(dT)20. The transcript levels of C*CD*s were analysed via RT-qPCR with Power SYBR™ Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) and the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems), according to the manufacturers' instructions. Transcript levels were calculated according to the ΔΔCq (formerly ΔΔCt) method (Livak and Schmittgen 2001) using the *Actin 4* (accession number: AB054978) and *Ubiquitin 3-3* (AB265782) genes as references (Higuchi et al. 2007; Yamada et al. 2007). The primers used in this experiment are listed in Supplementary Table S1. Statistical significance of the differential expression levels was assessed as previously described for three independent experiments (with mean centring and autoscaling) (Willems et al. 2008; Bustin et al. 2009). The Tukey–Kramer test at the 1% level and Student's *t* test were used for the tissue- and developmental stage-specific expression analysis and the analysis of carotenoid biosynthesis-related genes, respectively. The results are presented as the standardized mean of three independent experiments with the SE.

Vector construction

The backbone of the vector used for targeted mutagenesis was pDeCas9-Kan (modified form pDeCas9 (Fauser et al. 2014), where the *Bialaphos resistance* gene is replaced with *Neomycin phosphotransferase II* (*NPTII*)). Briefly, three pairs of complementary oligo DNAs for the *InCCD4* target sequences (Supplementary Table S1) were annealed at 95 °C for 5 min. The guide RNA (gRNA) and the target sequence-cloning vectors pMR203 (pENTR_L1L4_AtU6gRNA), pMR204 (pENTR_R4R3_AtU6gRNA) and pMR205 (pENTR_L3L2_AtU6gRNA) were digested with the restriction enzyme *Bbs*I and ligated to the annealed oligo DNA. The cloning vectors were verified by sequencing. The three gRNA expression cassettes were merged with the binary vector via an LR reaction. The destination vector was verified by *Eco*RI digestion.

Plant transformation

Rhizobium [*Agrobacterium*]-mediated transformation using an immature embryo-derived secondary embryo was performed as described previously (Kikuchi et al. 2005). For transformation, *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) strain LBA4404 harbouring a ternary plasmid for *virG* N54D (van der Fits et al. 2000) was used. The validity of the transformation was confirmed by PCR using total DNA extracted from young leaves and primers for *NPTII* (Supplementary Table S1). Total DNA was extracted from the young leaves of plants as previously described (Edwards et al. 1991). PCR was performed using GoTaq® Green Master Mix (Promega, Madison, WI, USA) in a thermal cycler, with initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 5 min.

Detection of mutants

First, we conducted a cleaved amplified polymorphic sequence (CAPS) analysis to detect mutations in the target region using the restriction enzyme *Acl*I and *InCCD4*-specific primers (Supplementary Table S1) as previously described (Watanabe et al. 2017). If targeted mutagenesis occurred, the restriction site would collapse, and the PCR-amplified DNA fragment would not be digested using restriction enzyme. Therefore, if mutagenesis occurred, the PCR fragments of non-digested and digested would be identical. Subsequently, the mutations were confirmed via DNA sequencing. DNA fragments of *InCCD4* were PCR-amplified using total DNA from transformants and the Advantage® 2 Polymerase Mix (BD Biosciences Clontech, Palo Alto, CA, USA) and the *InCCD4*-specific primers (Supplementary Table S1). PCR was performed using a thermal cycler, with initial denaturation at 95 °C for 1 min, followed by 30 cycles of 95 °C for 15 s and 68 °C for 30 s (two-step cycles), and an additional 10 min extension step at 70 °C for incorporation and preservation of 3′ A-overhangs. The PCR products were cloned into the pGEM®-T Easy Vector (Promega) and sequenced using an ABI 3130 Genetic analyzer (Life

Technologies) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Nucleotide and amino acid sequences were analysed using GENETYX-MAC (Software Kaihatsu Co.).

Carotenoid extraction and HPLC analysis

Carotenoids were extracted from petals and leaves, and analysed via HPLC as previously described (Kishimoto et al. 2007), with slight modifications. An acetone extract of frozen petals (0.3 g) and leaves (0.1 g) was partitioned between diethyl ether and aqueous NaCl. The organic layer was washed with 5.0 mM Tris–HCl (pH 8.0), and the residue was saponified with an equivalent amount of 10% KOH–MeOH for 1 h at room temperature. The saponified matter was then extracted with diethyl ether and washed with water. The organic layer was then dried and dissolved in 125 µL of MeOH and subjected to HPLC analyses. A non-saponified carotenoid extract was prepared according to the same method except for the saponification step. The total content of carotenoids was estimated from the absorbance at absorption maxima using the $E^{1\%}$ value of lutein (2550) (Britton 1995), defined as the theoretical absorbance of a 1% solution in a cell with a 10 mm path length. To identify carotenoids, the following carotenoid standards have been used: violaxanthin, neoxanthin (DHI lab products, Hørsholm, Denmark), lutein, β-carotene (Sigma-Aldrich, St. Louis, MO, USA), zeaxanthin and β-cryptoxanthin (Extrasynthese, Genay, France). The content of each carotenoid was calculated according to the total peak area of HPLC chromatograms at a wavelength of 450 nm, using program ChromNAV ver. 2 (Jasco, Tokyo, Japan). Measurements were performed in triplicate.

Results

Genome-wide identification of *I. nil CCD* genes

Searches for putative *CCD* homologous genes in the predicted transcripts from the *I. nil* genome sequence (Hoshino et al. 2016) and EST clones (http://ipomoeanil.nibb.ac.jp/) revealed a total of seven sequences representing the *I. nil CCD* gene subfamily. These *CCD* genes were designated *InCCD1a*, *InCCD1b*, *InCCD4*, *InCCD7*, *InCCD8*, *InCCD-like-a*, and *InCCD-like-b* based on their homology to tomato and Arabidopsis genes (Table 1). The numbers of pseudo-chromosomes, putative gene length, intron numbers and amino acid length are also listed in Table 1. Predicted amino acid sequences were used for phylogenetic analysis (Fig. 1).

Tissue- and developmental stage-specific expression of *InCCD*

Next, we analysed the tissue- and developmental stage-specific transcriptional levels of seven *InCCD*s via RT-qPCR (Fig. 2 and Supplementary Fig. S1). *InCCD1a*, *1b*, *like-a* and *like-b* were expressed almost constitutively, and their levels showed no significant difference between the different developmental stages of petals. In the roots, *InCCD7* and *8* were expressed at relative high levels, and no significant difference existed between the stages of petal development. The

Table 1 The *InCCD* genes and properties of the deduced proteins

Name	Description	Pseudo chromosome	Gene length (bp)[a]	Number of intron	Predicted protein length (AA)	Accession number
CCD1a	*Carotenoid cleavage dioxygenase 1a*	4	13,279	13	603	BR001460
CCD1b	*Carotenoid cleavage dioxygenase 1b*	12	7270	14	578	BR001461
CCD4	*Carotenoid cleavage dioxygenase 4*	15	3373	1	591	BR001462
CCD7	*Carotenoid cleavage dioxygenase 7*	9	3009	6	614	BR001463
CCD8	*Carotenoid cleavage dioxygenase 8*	10	6411	5	561	BR001464
CCDlike a	*Carotenoid cleavage dioxygenase like a*	10	12,085	10	509	BR001465
CCDlike b	*Carotenoid cleavage dioxygenase like b*	15	33,696	13	604	BR001466

[a]Number of nucleotides from predicted transcription start site to end site, including intron(s), in base pairs (bp)

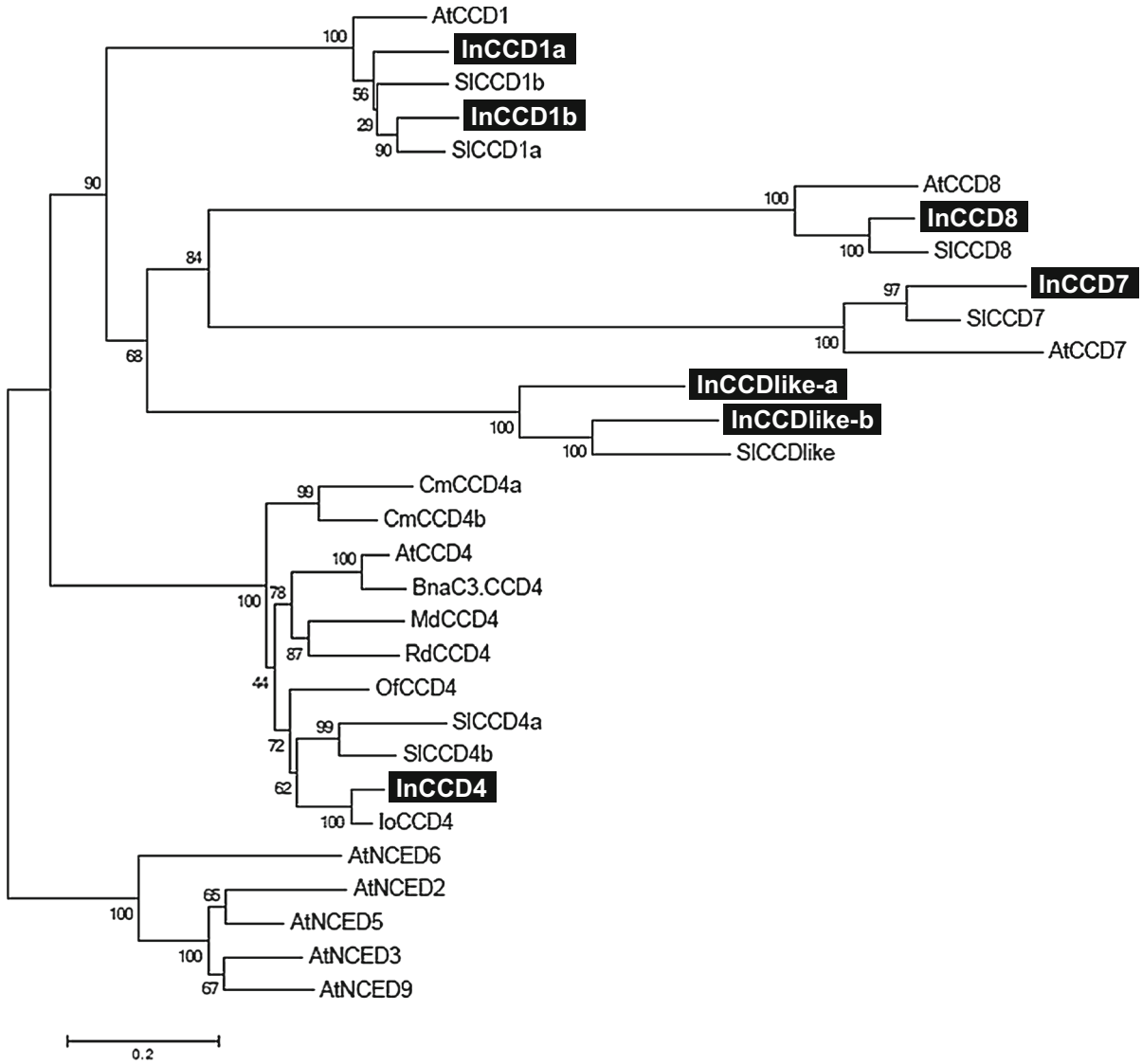

Fig. 1 Phylogenetic tree of CCDs of various plant species. The neighbor-joining phylogenetic tree (Saitou and Nei 1987) was generated based on the alignment of deduced protein sequences from CCD homologs in *I. nil* (black highlighted) and from various plant species. The horizontal branch length is proportional to the estimated number of amino acids substitutions per residue (Bar = 0.2 aa substitution per residue). Number at the branch points represents the bootstrap values for percentage of 1000 replicate trees. This is an unrooted tree. AtCCD1 (accession number: At3g63520), AtCCD4 (At4g19170), AtCCD7 (At2g44990), AtCCD8 (At4g32810), AtNCED2 (At4g18350), AtNCED3 (At3g14440), AtNCED5 (At1g30100), AtNCED6 (At3g24220), and AtNCED9 (At1g78390) are CCDs and NCEDs of Arabidopsis. SlCCD1a (Solyc01g087250), SlCCD1b (Solyc01g087260), SlCCD4a (Solyc08g075480), SlCCD4b (Solyc08g075490), SlCCD7 (Solyc01g090660), SlCCD8 (Solyc08g066650) and SlCCD-like (Solyc08g066720) are CCDs of tomato. CmCCD4a (AB247158) and CmCCD4b (AB247160) are CCDs of chrysanthemum. Other include IoCCD4 of *Ipomoea obscura* var. *lutea* (AB499059), MdCCD4 of apple (*Malus* × *domestica*, EU327777), OfCCD4 of osmanthus (*Osmanthus fragrans*, EU334434), RdCCD4 of rose (*Rosa* × *damascena*, EU334433) and BnaC3.CCD4 of rapeseed (*Brassica napus*, KP658825)

expression level of *InCCD4* was significantly higher in the cotyledons, leaves, and opened flowers than in the roots, stems and S1–S3 stages of petals. Moreover, among the tested *CCD* genes, only the expression of *InCCD4* increased significantly with floral tissue development, while carotenoid levels decreased with

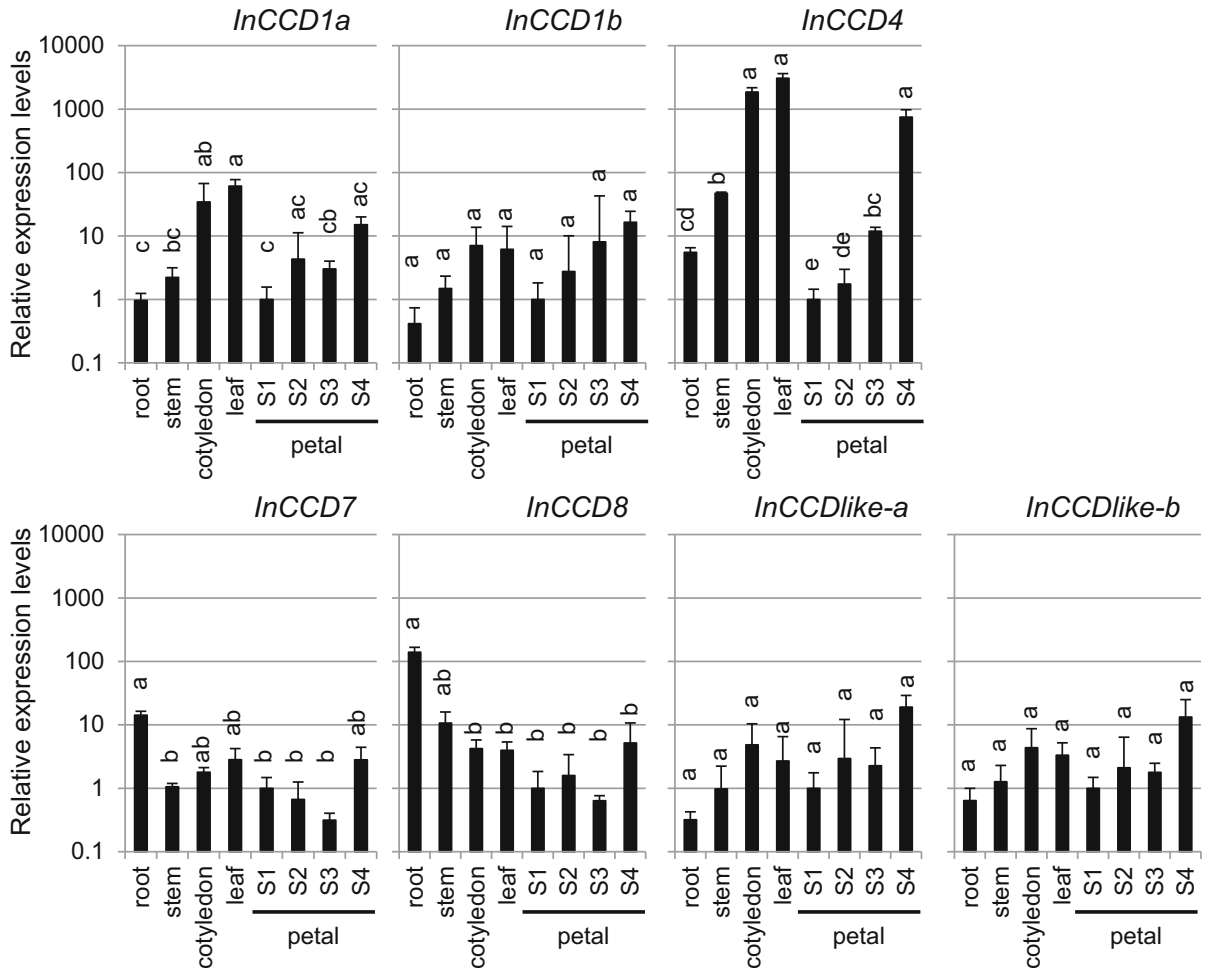

Fig. 2 Tissue- and developmental stage-specific expression levels of *InCCDs*. Quantitative real-time RT-PCR analysis was performed in biological triplicate using each *InCCD*-specific primers, and the expression levels were normalized against *actin* levels; mean values ± SE are shown ($n = 3$). Different letters within each tissue and stage indicate significant differences by Tukey–Kramer test ($P < 0.01$)

floral tissue development, as previously reported (Yamamizo et al. 2010). Therefore, we conducted a detailed analysis of InCCD4 amino acid sequence.

Amino acid sequence of InCCD4

Comparison of CCD4 sequences revealed that the InCCD4 protein contained four highly conserved His residues that have been previously described as typical ligands of a non-haem iron cofactor required for dioxygenase activity (Schwartz et al. 1997) (Fig. 3). Additionally, conserved Glu or Asp residues, which are used to fix iron-ligating His (Kloer and Schulz 2006), were also found in the InCCD4 protein sequence. Based on the results of amino acid sequence and tissue-specific gene expression analyses, we assumed that InCCD4 might exhibit conserved carotenoid cleavage activity and play some role in the petals of *I. nil* in the absence of carotenoids. Subsequently, *InCCD4* was knocked out through targeted mutagenesis to investigate InCCD4 function.

Knocking out *InCCD4* and identification of mutant plants

To completely inactivate InCCD4 in *I. nil*, we constructed CRISPR/Cas9 and gRNA vector for *InCCD4* (Supplementaly Fig. S2a) and introduced

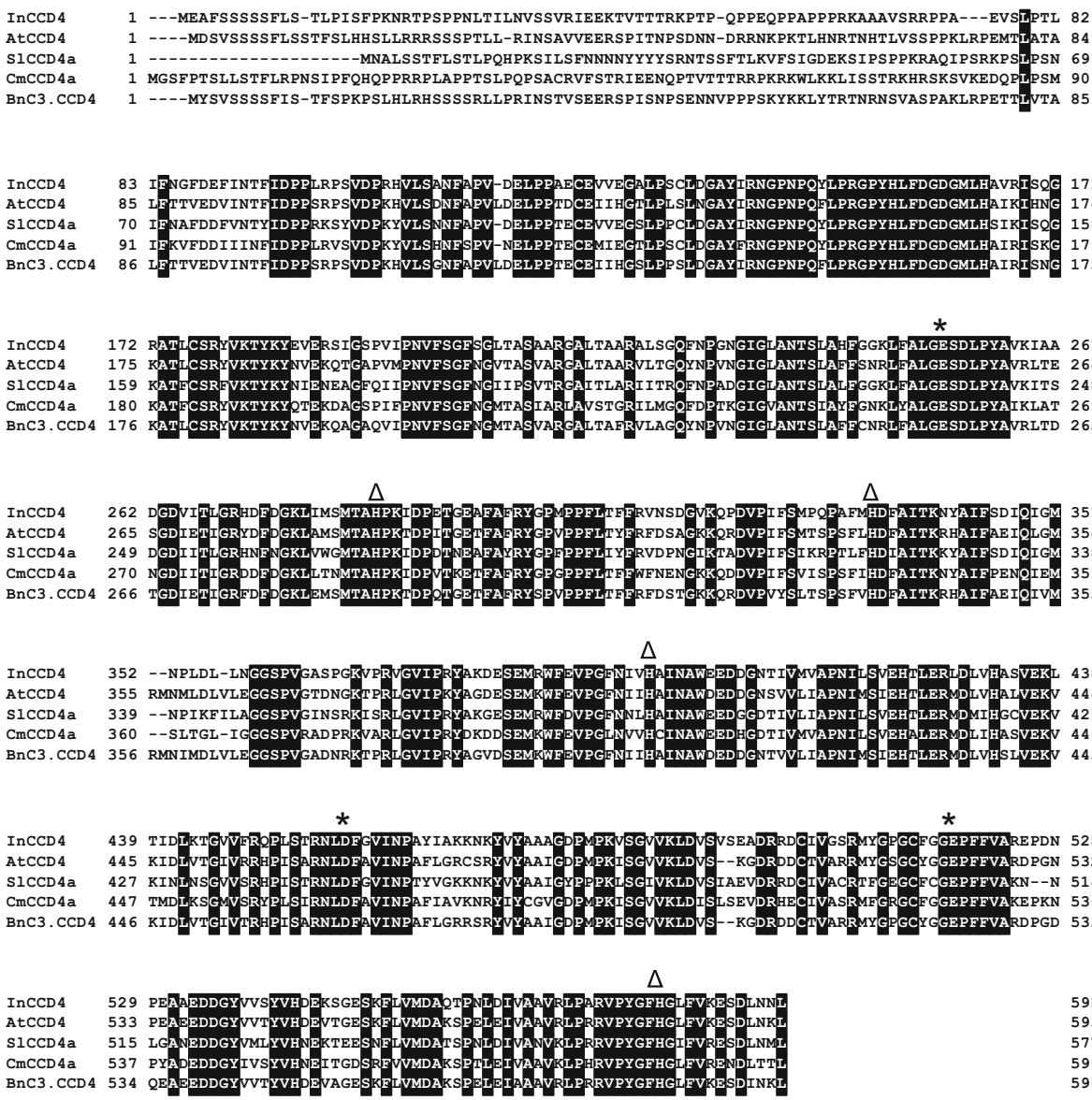

Fig. 3 Sequence comparison of CCD4 of various plant species. The amino acid sequences are given in the one-letter code and have been aligned, with the introduction of gaps (—), to maximize possible homology. Identical amino acids are indicated with black backgrounds. Conserved four His and three Glu or Asp residues are marked with triangles and asterisks respectively

this construct into *I. nil* cv. AK77 using *Agrobacterium*-mediated transformation. We designed three gRNAs (gRNA1, gRNA2, and gRNA3) with a target sequence within the first exon of the *InCCD4* gene in the genome to introduce mutations (Supplementary Fig. S2b). We designed the expected cleavage site of gRNA3 overlapping with the recognition sequence of the restriction enzyme *Acl*I to perform CAPS analysis. We searched the whole *I. nil* genome sequence to detect potential off-target sequences using the Web-based program GGGenome (http://gggnome.dbcls.jp/) and concluded that there were no potential off-target sites.

Nine lines of transgenic Cas9/gRNA plants were produced from independent transformation events (Supplementary Fig. S2c). CAPS analysis showed that five lines exhibited bi-allelic mutations in *InCCD4* alleles, because the PCR fragment of non-digested and digested fragment were identical (Supplementary Fig. S2d, e). All the bi-allelic *CCD4* mutants (*Inccd4*) showed pale yellow midribs, whereas no differences in petal colouration were observed between mono-allelic (or chimeric) mutants and non-transgenic (NT) plants. Subsequently, two independent *ccd4* lines were selected for further study. Sequence analysis showed that *ccd4* plants exhibited a frame-shift mutation in both alleles (Supplementary Fig. S3), and the putative amino acid sequences of the *ccd4* plants were truncated and incomplete (Supplementary Fig. S4).

Phenotypes and the amount and composition of carotenoids in *ccd4* mutant lines

Representative flower phenotypes of NT and the *ccd4* mutant line #11–1 and #14–1 are shown in Fig. 4a–f. The midribs of the flower petals of both *ccd4* plants showed pale yellow colouration (Fig. 4a–f). Apart from this petal colour change, no other morphological differences between NT and *ccd4* were visually distinguishable. The fertility of both mutants was normal, and unlike maize *vp14*, these plants did not show viviparity (Schwartz et al. 1997; Tan et al. 1997).

Subsequently, we compared the amount and composition of carotenoids in the petals of the *ccd4* mutant lines and NT. Representative HPLC chromatograms of saponified and non-saponified carotenoid extract are shown in Fig. 4g and Supplementary Fig. S5, respectively. These analyses enabled detection of the carotenoid composition and the existence of esterified carotenoids, respectively (Yamamizo et al. 2010). The total amount of carotenoids in the petals of the two *ccd4* lines was significantly increased (Fig. 4h). The carotenoid levels in the mutant lines were approximately 20 times higher than those in NT. However, the carotenoid components detected in the *ccd4* lines were almost the same as those in NT (Fig. 4g). Lutein accounted for the majority of the carotenoid components in both NT and the *ccd4* mutant lines. The HPLC chromatogram of the non-saponified carotenoid extract was almost identical to that of the saponified extract, indicating that the carotenoids present in the petals of transgenic plants were not esterified (Fig. 4g and Supplementary Fig. S5). In addition, only trace amount of chlorophyll a was detected in both NT and the *ccd4* mutant lines (Supplementary Fig. S5).

We also analysed the amount and composition of carotenoids in the leaves of progeny of the *ccd4* mutant lines and NT (Supplementary Fig. S6). The carotenoid components were almost same and no significant difference was detected in the carotenoid total amounts in the leaves. In addition, chromatograms of non-saponified extracts have peaks of chlorophylls (Supplementary Fig. S6b).

Expression of carotenoid biosynthetic genes in the petals of *ccd4* mutant lines

To investigate whether knockout of *InCCD4* affected the carotenoid biosynthesis pathway and related factors, we performed RT-qPCR to compare the expression levels of two carotenogenic and three genes involved in the carotenoid accumulation [*PSY*: AB499050, *CHYB*: AB499056, *Orange* (*Or*): LC314594, *Pale yellow petal 1* (*PYP1*), LC314595 and *Chromoplast-specific carotenoid-associated protein* (*CHRC*), LC314596] in the petals of opened flowers in the *ccd4* mutant lines (Fig. 5). *Or*, *PYP1*, and *CHRC* encode a post-translational stabilizer of PSY (Zhou et al. 2015; Park et al. 2016), a carotenoid esterase (Ariizumi et al. 2014), and a carotenoid-binding protein (Vishnevetsky et al. 1996; Leitner-Dagan et al. 2006), respectively. The expression levels of these genes showed no significant differences between the NT and *ccd4* plants; thus, we concluded that the increase in carotenoid levels was due solely to the loss-of-function of InCCD4.

Discussion

According to previous studies, the genome sequences of *CCD1* s, *CCD4* s, *CCD7*s, and *CCD8*s generally exhibit 11–13, 0–2, 5–6 and 6–7 introns, respectively (Ahrazem et al. 2010). The detected *CCD* sequences of *I. nil* nearly matched these patterns, suggesting that the *InCCD*s were correctly detected based on genomic information.

The expression level of *InCCD4* was significantly increased during floral tissue development, similar to what has previously been reported for *CmCCD4a* of

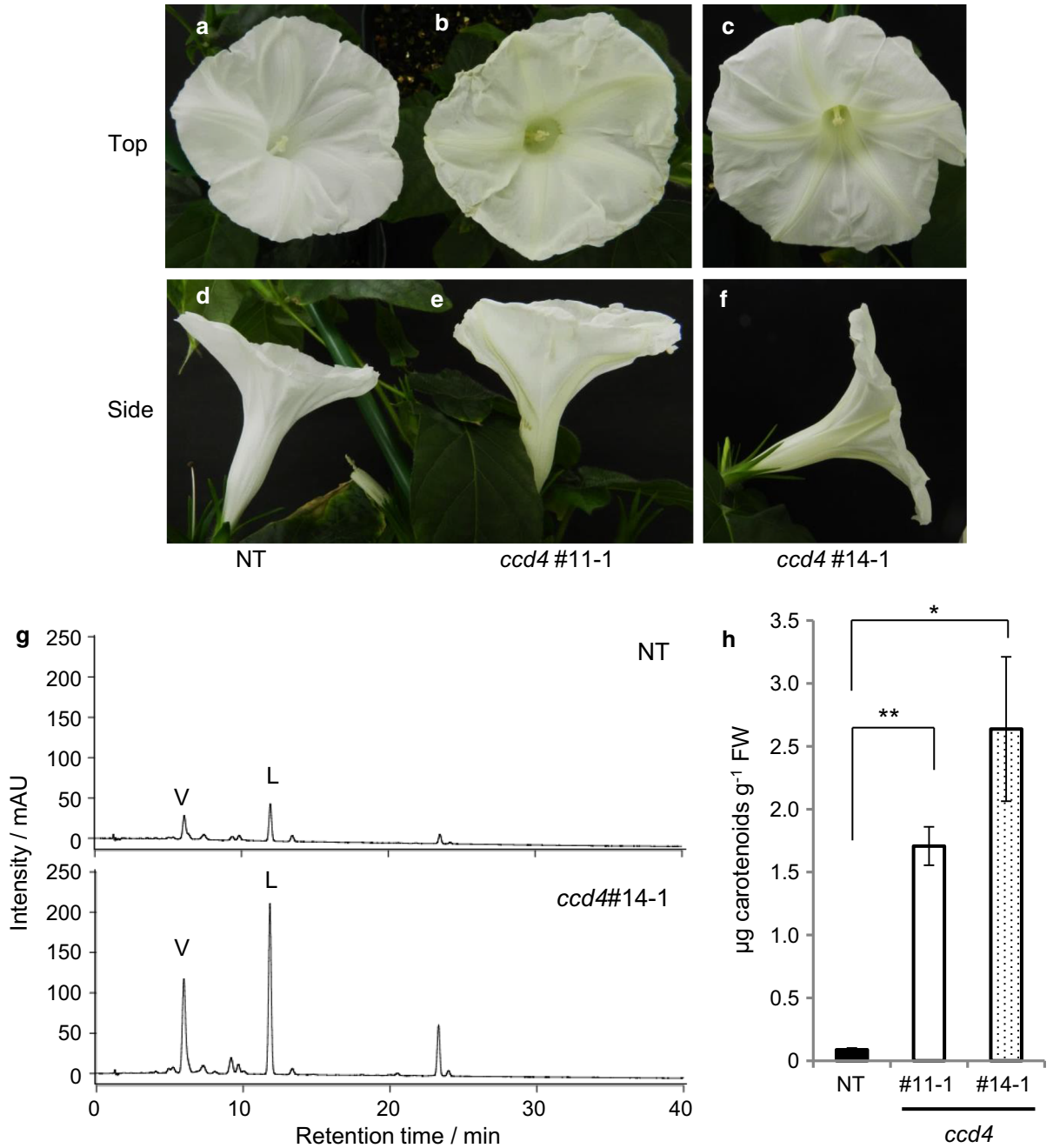

Fig. 4 HPLC chromatograms and total carotenoid concentration of flowers of non-transgenic (NT) and the *ccd4* plant. **a**–**f** Appearance of *ccd4* #11-1 (**b**, **e**), *ccd4* #14-1 (**c**, **f**) and non-transgenic (NT; **a**, **d**) opened flowers. Top: top view; Side: side view. **g** Representative HPLC elution profiles of non-saponified carotenoids extracted from petals of opened flower. V, violaxanthin; L, lutein; β, β-carotene. **h** Total carotenoid contents in the petal of opened flower of the NT and *ccd4*, as determined by HPLC analysis after saponification. All experiments were biologically repeated three times. Student's *t* test was used to determine statistical significance. Asterisks indicate significant difference ($*P < 0.05$, $**P < 0.01$) to NT. Error bars indicate ± SE ($n = 3$)

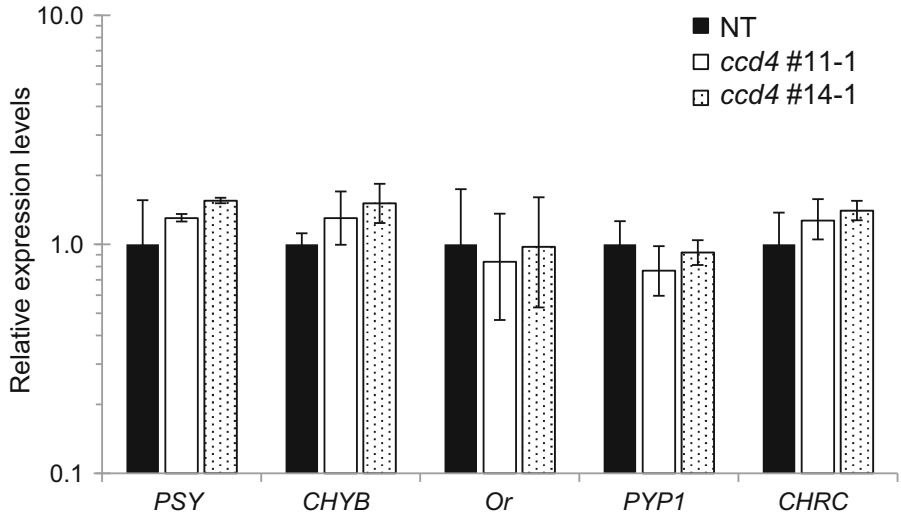

Fig. 5 The expression levels of carotenoid biosynthetic and accumulation related genes in the fully opened petal. Quantitative real-time RT-PCR analysis was performed in biological triplicate using each gene-specific primers, and the expression levels were normalized against *actin* levels; mean values ± SE are shown ($n = 3$). There was no significant difference between *ccd4*s and NT

chrysanthemum (Ohmiya et al. 2006). However, *InCCD4* was not exclusively expressed in floral tissue but also highly expressed in cotyledons and leaves. In a previous study, flower colour was found to be controlled by the *CCD4* family, which includes multiple *CCD4* genes, and at least one *CCD4* member is highly and exclusively expressed in flowers (Ohmiya et al. 2006; Rodrigo et al. 2013; Zhang et al. 2015). Moreover, only one homologous gene of *CCD4* was detected in the genomic sequence of *I. nil*. In most plants, the number of *CCD4* genes is variable between different species, with at least two genes being identified per species (Ahrazem et al. 2010; Vallabhaneni et al. 2010). The presence of a single *CCD4* gene has only been reported in a limited number of plant species with available genome sequences, such as Arabidopsis, papaya (*Carica papaya*), peach (*Prunus persica*) and sorghum (*Sorghum bicolor*) (Ahrazem et al. 2016), and *I. nil*. The results of the present study showed that it is not only *CCD4*s (which are exclusively expressed in petals) but also *InCCD4* (whose expression is not restricted to petals) that may contribute to flower colour.

According to previous studies in Arabidopsis, loss of AtCCD4 function alone does not affect carotenoid homeostasis; however, it is not only AtCCD4 but also AtCCD1 that contributes to carotenoid turnover (Gonzalez-Jorge et al. 2013; Lätari et al. 2015). Additionally, the observation that InCCD4 showed relatively high expression in the cotyledons and leaves of *I. nil* indicates that InCCD4 might affect carotenoid turnover together with InCCD1a and/or InCCD1b. However, as the carotenoid amounts and components in the leaves of the *ccd4* mutants were almost the same as those in NT, to analyse the effect of InCCD4 on carotenoid turnover, it will be necessary to knock out *InCCD1a* and *InCCD1b* and construct a double mutant, which is beyond the scope of the present study.

To elucidate the function of InCCD4, we generated *InCCD4* knockout mutants using the CRISPR/Cas9 system and obtained homozygous mutant lines. The putative amino acid sequence of InCCD4 in the *ccd4* plants was shortened and incomplete. Moreover, this sequence lacked the highly conserved His, Glu, and Asp residues (Tan et al. 2003; Kloer and Schulz 2006), indicating that the carotenoid cleavage activity of InCCD4 was lost in *ccd4* plants. The colour change of the midribs of the flowers of *ccd4* plants was very noticeable. Because the expression levels of carotenoid accumulation related genes showed no significant differences, loss of InCCD4 function increased carotenoid accumulation in the petals. Hence, we concluded that it is not only low carotenogenic gene expression (Yamamizo et al. 2010) but also the cleavage activity of InCCD4 that contributes to the control of carotenoid accumulation in floral tissues of *I. nil*.

Carotenoid levels were significantly increased in the petals of *ccd4* mutant lines and chlorophyll levels were almost identical in *ccd4* mutants and NT, so we concluded that yellow colouration was due to carotenoid accumulation. However, these components

did not differ from NT. The majority of the carotenoid components found in both NT and the *ccd4* mutants consisted of non-esterified (free form) lutein, violaxanthin, and β-carotene, although the β-carotene level was below the detection limit in NT, reflecting the fact that the level of carotenoid accumulation related gene expression in the petals of the *ccd4* mutants was the same as in NT, which was still lower than that in *I. obscura* var. *lutea* (Yamamizo et al. 2010). In a previous study (Watanabe et al. in press), we successfully changed the components and increased the total amount of carotenoids after overexpressing genes encoding carotenoid biosynthesis enzymes, including the rate-limiting enzyme PSY (Giuliano et al. 1993; Moehs et al. 2001) and key enzymes responsible for the addition of hydroxyl residues required for the esterification of carotenoids (CHYB) (Galpaz et al. 2006; Yamamizo et al. 2010). Thus, the combination of *InCCD4* mutagenesis and overexpression of carotenoid biosynthetic and accumulation related genes sufficiently would increase the carotenoid content in the petals of *I. nil* to produce yellow petals.

The carotenoids contained in the petals of *ccd4* plants were not esterified and existed in the free form. Esterified carotenoids are the dominant chemical form for the storage of these compounds within chromoplasts. In the petals of *I. obscura* var. *lutea*, β-carotene derivatives (β-carotene, β-cryptoxanthin, and zeaxanthin) have been shown to account for approximately 85%, and xanthophylls such as β-cryptoxanthin and zeaxanthin exist in esterified forms (Yamamizo et al. 2010). We assume that low levels of carotenoids observed in the petals of the *ccd4* mutant lines partially reflect the absence of esterification activity. Therefore, promotion of esterification activity will increase carotenoid accumulation. Overexpression of *PYP1*, which encodes the enzyme that esterifies carotenoids (Ariizumi et al. 2014), may enhance esterification activity and could increase carotenoid accumulation; experiments aimed at examining this possibility are currently underway.

In the present study, we focused on the function of the InCCD4 enzyme in the petals of *I. nil*, which can store only trace amounts of carotenoids. Then we successfully increased carotenoid accumulation in the petals changed their colour. These results suggest that the low carotenoid levels observed in the floral tissues of *I. nil* are due to not only low carotenogenic gene expression but also carotenoid degradation through InCCD4. Thus, knocking out *CCD4* will produce novel flower colours and contribute to the development of nutritionally improved agricultural crops and fruits with high economic value, such as peaches and bananas (Brandi et al. 2011; Bai et al. 2016; Buah et al. 2016). The present study represents an important milestone towards the application of the CRISPR/Cas9 system to modify flower colour via increasing the amounts of carotenoids in petals lacking the ability to accumulate carotenoids and to further carotenoid metabolic engineering.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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