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ORIGINAL ARTICLE

In planta cleavage of carotenoids by *Arabidopsis* carotenoid cleavage dioxygenase 4 in transgenic rice plants

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Abstract A family of carotenoid cleavage dioxygenases (CCDs) produces diverse apocarotenoid compounds via the oxidative cleavage of carotenoids as substrates. Their types are highly dependent on the action of the CCD family to cleave the double bonds at the specific position on the carotenoids. Here, we report in vivo function of the *AtCCD4* gene, one of the nine members of the *Arabidopsis* CCD gene family, in transgenic rice plants. Using two independent single-copy rice lines overexpressing the *AtCCD4* transgene, the targeted analysis for carotenoids and apocarotenoids showed the markedly lowered levels of β-carotene (74 %) and lutein (72 %) along with the changed levels of two β-carotene (C_{40}) cleavage products, a two-fold increase of β-ionone (C_{13}) and de novo generation of β-cyclocitral (C_{10}) at lower levels, compared with non-transgenic rice plants. It suggests that β-carotene could be the principal substrate being cleaved at 9–10 (9′–10′) for β-ionone and 7–8 (7′–8′) positions for β-cyclocitral by AtCCD4. This study is *in planta* report on the generation of apocarotenal volatiles from carotenoid substrates via cleavage by AtCCD4. We further verified that the production of these volatiles was due to the action of exogenous AtCCD4 and not the expression of endogenous rice CCD genes (*OsCCD1*, *4a*, and *4b*).

Keywords Apocarotenoid · *Arabidopsis* · Carotenoid · Carotenoid cleavage dioxygenase · Rice · *Oryza sativa* L.

M.-H. Song and S.-H. Lim contributed equally and should be considered co-first authors.

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Introduction

Carotenoid cleavage products (CCPs) are apocarotenoid compounds derived from carotenoid substrates by catalysis through the carotenoid cleavage dioxygenases (CCDs). CCPs have biologically diverse functions in plants, acting as hormones, pigments, flavors, and defense compounds (Walter et al. 2010). Plant CCDs are encoded as a multigene family and cleave diverse carotenoids at different double bond positions with specificity (Auldridge et al. 2006b; Walter and Strack 2011). The *Arabidopsis* CCD gene family consists of nine members, four CCDs (1, 4, 7, and 8) and five 9-*cis*-epoxycarotenoid dioxygenases (NCED 2, 3, 5, 6, and 9). The latter five NCEDs are involved in the production of xanthoxin (C_{15}) as a precursor of abscisic acid by oxidative cleavage at the 11–12 double bonds of the 9-cis-epoxycarotenoid substrates (C_{40})

neoxanthin and violaxanthin. This affects seed dormancy, water stress tolerance, and plant growth (Toh et al. 2008; Martinez-Andujar et al. 2011; Frey et al. 2012). The function of the former four CCDs is predicted to differ from that of the NCEDs in *Arabidopsis* (Walter and Strack 2011).

The enzymes AtCCD7 and AtCCD8 have been characterized in studies of the more axillary branching mutant (*max*) to produce novel signaling molecules. These molecules inhibit lateral shoot branching through the sequential cleavage of AtCCD7/MAX3 and AtCCD8/MAX4, which are related to the strigolactone biosynthetic pathway (Sorefan et al. 2003; Booker et al. 2004; Umehara et al. 2008). In addition, AtCCD1 has been demonstrated in vitro to generate various flavor and aromatic compounds via the cleavage of multiple carotenoid substrates at double bonds in a non-specific manner (Schwartz et al. 2001; Schmidt et al. 2006; Vogel et al. 2008). In the case of the *in planta* cleavage activity of AtCCD1, loss of function of this gene increases the carotenoid levels in seeds (Auldridge et al. 2006a) and the overexpression of *AtCCD1* endows insect resistance in *Arabidopsis* by generating β-ionone (Wei et al. 2011). Similarly, CCD1 has also been reported to produce potent scent, flavor, and aroma compounds in the flowers and fruits of petunia, tomato, grape, and *Osmanthus fragrans* Lour. (Simkin et al. 2004a; Simkin et al. 2004b; Mathieu et al. 2005; Baldermann et al. 2010).

In comparison with the eight CCDs discussed above, CCD4 studies have mainly reported a role for CCD4 in white color formation via carotenoid pigment cleavage during flower development in chrysanthemum (*Chrysanthemum morifolium*, CmCCD4a) and *Lilium brownii* var. *colchesteri* (LbCCD4) (Ohmiya et al. 2006; Hai et al. 2012). In addition, white-fleshed mutants of potato (*Solanum tuberosum* cv Desiree and cv Maris Píper) and peach (*Prunus persica* L. Batsch. cv Redhaven Bianca) displayed elevated expression of *CCD4* genes during tuber and fruit development when compared with yellow-fleshed cultivars of potato (*Solanum tuberosum* cv 333-16 and cv Mayan Gold) and peach (*Prunus persica* L. Batsch. cv Redhaven), respectively (Campbell et al. 2010; Brandi et al. 2011; Bai et al. 2016). Two types of CsCCD4a/4b, encoded by crocus *CCD4* genes, showed direct cleavage activity of β-carotene at the 9–10 (9′–10′) positions during stigma development, releasing β-ionone (Rubio et al. 2008; Ahrazem et al. 2010). Additional cleavage functions of CCD4 were characterized in vitro using *AtCCD4*, *MdCCD4*, *CmCCD4a*, *RdCCD4*, and *OfCCD4* genes cloned from *Arabidopsis*, apple (*Malus domestica*), chrysanthemum, rose (*Rosa damascene*), and osmanthus (*Osmanthus fragrans*), respectively (Huang et al. 2009). At this time, AtCCD4 activity was believed to produce β-ionone from 8′-apo-β-carotene-8′-al when expressed not in plants, but in *E. coli* (Huang et al. 2009). Recently, endogenous *AtCCD4* was suggested via linkage mapping and genome-wide association studies to play a major role in β-carotene degradation in drying seeds and senescing leaves of *Arabidopsis*, without showing CCPs from AtCCD4 action (Gonzalez-Jorge et al. 2013). In this study, we characterized the *in planta* function of *AtCCD4* through its constitutive overexpression in transgenic rice plants. Our investigation may aid in the understanding of the flux of the pathways from carotenoids to diverse CCPs.

Materials and methods

Vector construction and rice transformation

A 1,788 bp genomic fragment encoding *AtCCD4* was PCR-amplified from *Arabidopsis* (*Arabidopsis thaliana* ecotype Col-0) with specific primer sets (5′-AAAAAGCAGGCT CAATGGACTCTGTTTC-3′/5′-AGAAAGCTGGGTGTT AAAGCTTATTAAGGTC-3′) as part of a Gateway cloning strategy (Karimi et al. 2002). The amplified product was subcloned into pDONR221 (Invitrogen, Carlsbad, CA) and the final vector of *pPGD1::AtCCD4* was prepared by further recombination with the destination vector *p600-PGD1* that was equipped with a 5′-upstream region of the rice phosphogluconate dehydrogenase 1 (PGD1) gene as a constitutive PGD1 promoter (Park et al. 2010). The *E. coli* strain containing the *pPGD1::AtCCD4* construct was triparentally mated with *Agrobacterium* LBA 4404 (harboring the *pSB* vector) and the conjugal helper strain HB101 (harboring the *pRK2013* plasmid). The resultant *Agrobacterium* strain was co-cultivated with embryogenic calli proliferated from mature seeds of japonica-type Korean rice (*Oryza sativa* L., cv. Hwayoung). Transgenic rice plants were regenerated from these transformed calli via shooting and rooting procedures on selection medium containing phosphinothricin (4 mg/L) and cefotaxime (500 mg/L) and subsequently grown in a greenhouse until maturity (Park et al. 2015).

Genomic DNA analysis

Genomic DNAs were extracted from transgenic rice leaf tissues using a modified cetyltriammonium bromide method. To estimate the copy number, genomic PCRs were performed TaqMan-PCRs were performed using assay mixtures with primers/probe sets of NOS terminator (tNOS) as a target gene region and α-tubulin as an internal reference. The tNOS assay was performed with a pair of primers and a probe—tNos-fw, 5′-GCATGACGTTATT TATGAGATGGGTTTT-3′/tNos-rv, 5′-CGATAGAAAAC AAAATATAGCGCGCA-3′; tNos-probe, 5′-TAGACTCC

CGCAATTAT-3—and labeled with the fluorescent reporter dye 6-carboxy-fluoroscein (FAM). The α-tubulin assay, involving α-tubulin labeled with the fluorescent reporter dye VIC, was commercially provided (Assay ID: Os03643486_s1; Applied Biosystems, Foster City, CA). TaqMan PCR was carried out using a Mx3000p Real-Time PCR machine (Stratagene, La Jolla, CA) in a 20 µl reaction mixture contained 50 ng DNA, 1 µl tNOS, and 1 µl α-tubulin per assay, and 10 µl 2× TaqMan Gene expression master mix (ID: 4369016; Applied Biosystems). The amplification conditions consisted of 1 cycle of 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 58 °C for 40 s, and 68 °C for 1 min. The results were calculated with the single-copy reference of tNOS introgression from the homozygous transgenic rice line PAC 4, which was assigned a value of 1 as a positive control (Ha et al. 2010).

For Southern blot analyses, 20 µg of DNA was digested with *Sca*I restriction enzyme. Digested DNA was separated on a 1 % agarose gel and transferred onto a Hybond N+ nylon membrane (Amersham Pharmacia, Uppsala, Sweden). The membrane was hybridized with a probe to detect the PGD1 promoter of the vector construct. This probe was amplified with specific forward (5′-GGGGATATTTGGTG GGCTAT-3′) and reverse (5′-GCATGCTGTCGAAGAA ACAA-3′) primers using a PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany). Hybridization and detection were performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) and the membrane was then exposed to Lumi-Film Chemiluminescent Detection film (Roche) for 12 h and developed.

RNA analysis

Total RNAs were extracted from leaf tissues using a Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). The first-strand cDNAs were synthesized using an mRNA Selective PCR kit (Takara, Tokyo, Japan). For semiquantitative reverse transcriptase-PCR (RT-PCR), each reaction containing 1 µl of the cDNA template was amplified under the condition of 1 cycle of at 94 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min/kb followed by 10 min at 72 °C using rTaq DNA Polymerase (Takara). For quantitative real-time (qRT)-PCR, the PCR mixture contained 0.25 µl cDNA, 10 µl Thunderbird™ SYBR qPCR Mix (Toyobo, Osaka, Japan), and 200 nM of each gene-specific primer pair in a final volume of 20 µl. The qRT-PCR was performed using a Bio-Rad CFX96 Real-time System (Bio-Rad Laboratories, Hercules, CA) under the following conditions: 1 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The specificity of the PCR amplification was checked with melting curve analysis (60–95 °C) after 40 cycles. For each sample, threshold cycles (Ct; the cycle at which the increase in fluorescence exceeded the threshold setting) were automatically determined. The expression level was analyzed with CFX manager™ ver. 2.1 (Bio-Rad Laboratories), and relative quantification of gene expression was performed using *OsUbiquitin5* (*OsUBQ5*) as a reference. First RT-PCRs to select the *AtCCD4*-overexpressed transgenic rice plants were amplified with a *lamda* phage site-specific primer set of *att*B1 and *att*B2 used in Gateway vector cloning (Karimi et al. 2002) and a *AtCCD4*-specific primer set used in *AtCCD4* vector cloning above. The gene-specific primer pairs for further RT-PCR and qRT-PCR to compare the transcript levels are listed in Table 1. All reactions were performed with three technical replicates.

Carotenoid and chlorophyll analysis

Rice leaves were ground to a fine powder using liquid nitrogen and stored at −80 °C before extraction. The extraction method used for carotenoid analysis was similar to that described in Kim et al. (2010). Briefly, we extracted carotenoids from the rice leaf samples (100 mg) by adding 3 ml of ethanol containing 0.1 % ascorbic acid (w/v), vortex mixing for 20 s, and by placing the mixture in a water bath at 85 °C for 5 min. After a saponification procedure in which the carotenoid extract was treated with potassium hydroxide (120 µl, 80 %, w/v) in the 85 °C water bath for 10 min, extract samples were placed immediately on ice and cold deionized water (1.5 mL) was added with β-apo-8′-carotenal (0.05 mL, 25 µg/mL) as an internal standard. Carotenoids were extracted twice with hexan (1.5 mL) by centrifugation at 1200 *g* to separate the layers. Aliquots of the extracts were dried under a stream of nitrogen and redissolved in 50:50 (v/v) dichloromethane/methanol before HPLC analysis (Kim et al. 2015).

The carotenoids were separated on a C30 YMC column (250 × 4.6 mm, 3 µm; Waters Corporation, Milford, MA) by HPLC as described above. Chromatograms were generated at 450 nm. Solvent A consisted of methanol/water (92.8, v/v) with 10 mM ammonium acetate. Solvent B consisted of 100 % methyl *tert*-butyl ether. Gradient elution was performed at 1 mL/min under the following conditions: 0 min, 83 % A/17 % B; 23 min, 70 % A/30 % B; 29 min, 59 % A/41 % B; 35 min, 30 % A/70 % B; 40 min, 30 % A/70 % B; 44 min, 83 % A/17 % B; 55 min, 83 % A/17 % B. For quantification purposes, calibration curves were drawn by plotting four different concentrations of carotenoid standards according to the peak area ratios with β-apo-8′-carotenal. The carotenoid levels were estimated using HPLC, and identification and peak assignment were primarily based on a comparison of their retention time and UV–vis spectra. A β-apo-8′-carotenal was

Table 1 Primer sets used for quantitative real-time (qRT)-PCR and semiquantitative reverse transcriptase (RT)-PCR to confirm the expression levels in rice leaf tissues

Gene	Accession number or Locus ID	Primer sequence (forward/reverse)	Product size (bp)
qRT-PCR			
AtCCD4	At4g19170	5′-GGATTCAATATCATTCACGCTATT-3′/5′-TCTTCACCTTCTCCACCAA-3′	114
OsCCD1	Os12g0640600 (Os12g44310)	5′-TCTGGAGAAGAAGATGATGGTTAT-3′/ 5′-GGGAACTCGGCTTGGTAG-3′	138
OsCCD4a	Os02g0704000 (Os02g47510)	5′-CGGGCTTCAACATCATGC-3′/5′-TAGCTCCATGTGCTCCAG-3′	116
OsCCD4b	Os12g0435200 (Os12g24800)	5′-TTGATCTCGTCGGCTCTG-3′/5′-ACCACTATCCTCGTTTCCATAC-3′	122
OsUBQ5	Os01g0328400 (Os01g22490)	5′-GAAGTAAGGAAGGAGGAGGA-3′/5′-AAGGTGTTCAGTTCCAAGG-3′	100
Semiquantitative RT-PCR			
AtCCD4	At4g19170	5′-CCGTCGTTTCTCCATGACTT-3′/5′-AAATCGAGATTCCTCGCTGA-3′	401
OsUBQ5	Os01g0328400 (Os01g22490)	5′-GTCCTCAGCCATGGAGC-3′/5′-GGACACAATGATTAGGG-3′	242

purchased from Sigma Chemical Company (St. Louis, MO). The α-carotene, β-carotene, lutein, zeaxanthin, antheraxanthin, and violaxanthin were purchased from CaroteNature GmbH (Lupsingen, Switzerland).

To extract total chlorophylls, the same rice leaf powders were mixed at 500 rpm in 100 % methanol at 70 °C for 30 min with a Thermomixer Comfort (model 5355, Eppendorf AG, Hamburg, Germany) and centrifuged at 800 g and 4 °C for 10 min. Absorbance of supernatant was measured with a spectrophotometer (Optizen POP, Mecasys Co., Daejeon, Republic of Korea) at 666 and 653 nm. The total content of chlorophylls was calculated according to Wellburn's equation (1994).

Carotenoid cleavage product extraction and GC–MS analysis

We extracted CCPs from 150 mg of leaves by adding 1 ml ethanol, sonicating for 5 min, vortex mixing for 3 min, and incubating at 20 °C in a shaker. After centrifugation at 10,000 rpm, the supernatant was collected and concentrated under room temperature. The resulting residues were redissolved in chloroform in a final volume of 75 µl. Analyses were performed using an Agilent gas chromatography time-of-flight mass spectrometry (GC–TOF–MS) system (7890A) with an autosampler (7693) (Agilent Technologies, Santa Clara, CA) equipped with a Rtx-5MS capillary column (30 m length × 0.25 mm i.d. × 0.25 µM film thickness; Agilent J&W GC column). The injector temperature was 250 °C and the injection volume was 1 µL. The oven temperature program commenced from 50 °C for 2 min, followed by a 5 min hold at 180 °C (15 °C/min), and finally a hold of 3 min, with a transfer line temperature of 260 °C (15 °C/min). In the MS, the ionization was at −70 V (electron energy) with a source temperature of 220 °C. The detector voltage was 1850 V and the mass range was set at 50–600 m/z with an acquisition rate of 10 spectra per second. Apocarotenoid standards for α-ionone, β-ionone, pseudoionone, 6-methyl-5-hepten-2-one (MHO), and β-cyclocitral were purchased from Sigma.

Results

Integration and overexpression of the AtCCD4 gene in transgenic rice plants

To introduce and express the *AtCCD4* gene in rice plants, a *p600-PGD1* vector harboring the *AtCCD4* gene under the control of the *PGD1* promoter (Park et al. 2010) was constructed. Introduction of the exogenous *AtCCD4* gene was first confirmed by expression analysis with two cloning primer pairs in leaf tissues of six transgenic rice plants at the T_0 generation (Supplementary Fig. S1). To verify the transgene integration and determine the copy number at the same T_0 generation (Fig. 1), TaqMan PCR results showed that the single-copy insertion of the heterozygous transgene had a value of 0.5 in the T_0 generation, calculated by comparison with the single-copy reference of tNOS introgression from the homozygous transgenic rice line PAC 4 (Ha et al. 2010), which was assigned a value of 1 as a positive control (Fig. 1a). Southern blot analysis also confirmed the single-copy insertion of the transgene by displaying two signal bands, one of which was a 1.6 kb band generated from the endogenous PGD1 promoter region on the rice genome as an internal control when hybridized with a PGD1 promoter-specific probe because we used the rice PGD1 promoter to induce the constitutive overexpression of *AtCCD4* (Fig. 1b). Two independent *AtCCD4*-transgenic rice lines 1 (T-1) and 5 (T-5) were finally selected as the single-copy events (Fig. 1).

To determine the expression levels of the *AtCCD4* transgenes in rice plants, semiquantitative RT-PCR

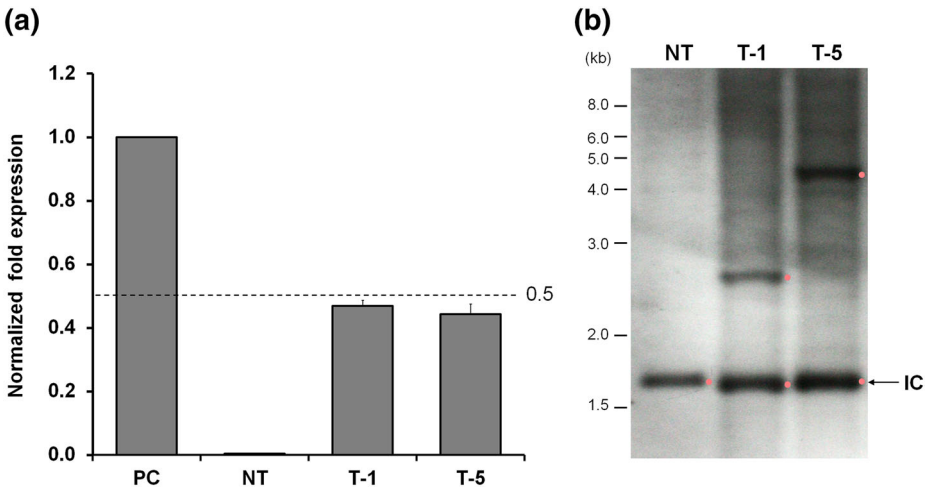

Fig. 1 TaqMan PCR (**a**) and genomic Southern blotting (**b**) analyses to determine the insertion and copy number of the *AtCCD4* transgene in the T_0 generation of transgenic rice plants. TaqMan PCR was performed in the NOS terminator region with α-tubulin as a reference. Genomic DNA digested with *Sca*I was hybridized with a probe of the PGD1 promoter for Southern blot analysis. IC means internal control, which was a 1.6 kb signal generated from the endogenous PGD1 promoter region in the rice genome. PC indicates the transgenic rice event PAC 4 (Ha et al. 2010) that was previously confirmed to have a single copy of the NOS terminator as a homozygous transgene line. NT means non-transgenic rice plants

(Fig. 2a) and qRT-PCR (Fig. 2b) were performed using 2-month-old leaves of T_1 plants. All three sibling lines from the two independent transgenic lines T-1 and T-5 showed stable overexpression of the *AtCCD4* transgene when compared with non-transgenic (NT) control rice plants (Fig. 2). Their visual phenotype, including plant height and fertility, looked similar to that of the NT control rice.

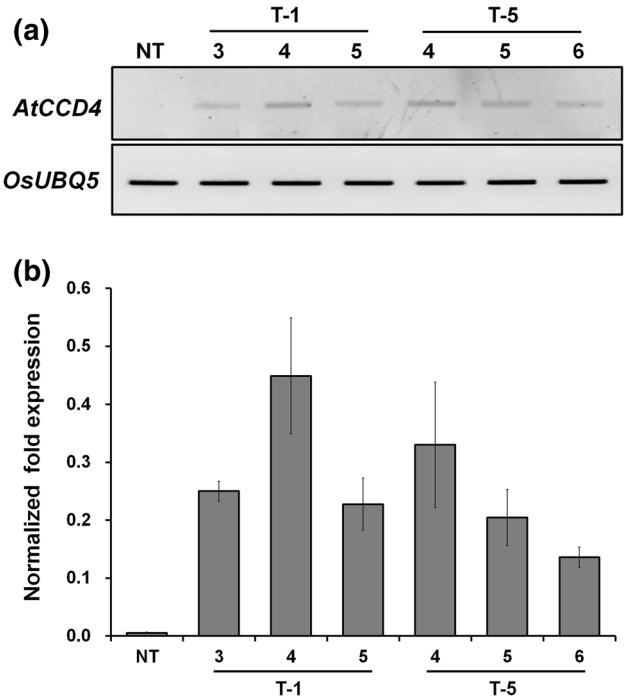

Fig. 2 Semiquantitative RT-PCR (**a**) and quantitative RT-PCR (**b**) analyses to confirm the transcript levels of the *AtCCD4* transgene in the leaf tissues of transgenic rice plants. The rice ubiquitin 5 (*OsUBQ5*) gene was used for normalization of the amounts of leaf RNA. NT denotes non-transgenic rice plants. Each value was the mean of three replicates

Reduced carotenoid levels in AtCCD4-overexpressing transgenic rice plants

To investigate the carotenoid cleavage activity of AtCCD4, the total content and compositions of the carotenoids were analyzed in the same leaf samples used for expression analysis (Fig. 3). Six carotenoids, namely α-carotene, β-carotene, lutein, zeaxanthin, antheraxanthin, and violaxanthin, were detected, with major levels of β-carotene and lutein. Transgenic lines showed a decrease in total carotenoid levels (an average of 130.59 ng/μl), indicating a reduction of 26 % compared with NT rice (an average of 176.04 ng/μl). In particular, two major carotenoids (β-carotene and lutein) and two minor carotenoids (α-carotene and violaxanthin) showed lower average amounts of 62.53 ng/μl (a 26 % reduction), 59.26 ng/μl (a 28 % reduction), 0.93 ng/μl (a 49 % reduction), and 3.45 ng/μl (a 22 % reduction) in transgenic leaf tissues than the average amounts of 84.47, 82.76, 1.83, and 4.43 ng/μl in NT lines, respectively (Fig. 3a and Supplementary Table S1). In contrast, zeaxanthin and antheraxanthin showed a small increase in the transgenic plants. No proportion of each carotenoid significantly differed because the change in the total amount was due to the decrease in the two major carotenoids (Fig. 3b and Supplementary Table S1). Thus, we speculated that AtCCD4 may influence the breakdown of four carotenoids (β-

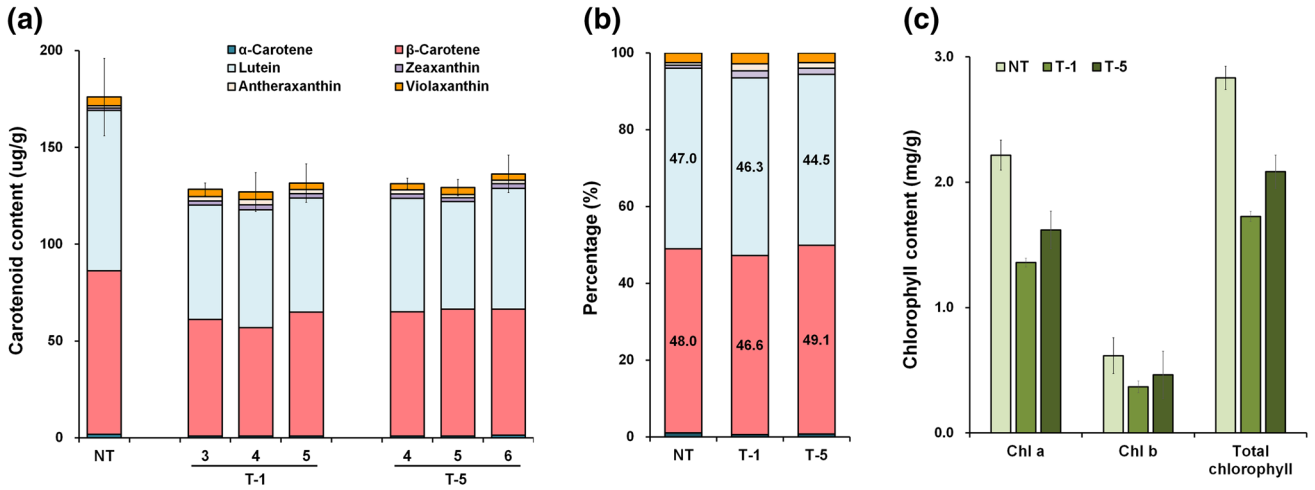

Fig. 3 Levels of carotenoids and chlorophylls. Contents (**a**) and composition (**b**) of carotenoids and contents of chlorophylls (**c**) were analyzed with the same leaf samples used for gene expression analyses. Total carotenoid levels were calculated as the sum of six carotenoid subtype levels (α-carotene, β-carotene, lutein, zeaxanthin, antheraxanthin, and violaxanthin). Values of carotenoids (μg/g flesh weight) and chlorophylls (mg/g flesh weight) represent the means of three and four replicates with the standard error (SE), respectively. More detailed results of the carotenoid composition are given in Supplementary Table S1

carotene, lutein, α-carotene, and violaxanthin) in transgenic rice plants and might directly and/or indirectly use β-carotene and lutein as the main cleavage substrates. The decreased leaf carotenoids in two transgenic lines affected the decline of total chlorophyll contents to 1.73 and 2.08 from 2.83 (mg/g FW) of NT rice (Fig. 3c). It was a plausible result considering the close correlation between two components of carotenoids and chlorophylls because leaf carotenoids are coexisted with chlorophylls in the leaf chloroplasts.

Detection of CCPs in AtCCD4-overexpressing transgenic rice plants

To determine the carotenoid cleavage activity of AtCCD4, apocarotenoid analysis was performed with GC-TOF–MS using 2-month-old leaves from homozygous T_4 plants of the *AtCCD4* transgenic rice line 1 (T-1) (Fig. 4). Of the five standard CCP compounds used for targeted analysis, three CCPs—α-ionone, pseudoionone, and MHO—were not detected in either NT control or *AtCCD4* transgenic rice. Of the remaining two apocarotenoids detected (Fig. 4a), β-ionone was observed with a two-fold higher level in *AtCCD4* transgenic rice than in NT rice and β-cyclocitral was newly observed in *AtCCD4* transgenic rice, but not in NT rice (Fig. 4b). Given that β-ionone is a cleavage product of the 9–10 (9′–10′) double bonds of β-carotene and α-carotene and β-cyclocitral is a cleavage product of the 7–8 (7′–8′)7–8 (7′–8′) double bond of β-carotene, these results strongly support the data suggesting that β-carotene is the preferred substrate and α-carotene could also become a substrate for exogenous AtCCD4 activity. These results are consistent with the lower amounts of β-carotene (62.53 ng/μl) and α-carotene (0.93 ng/μl) in *AtCCD4* transgenic plants than in NT plants (84.47 and 1.83 ng/μl, respectively) (Fig. 3a).

Expression levels of rice CCD genes in AtCCD4-overexpressing transgenic rice plants

To verify whether the reduction in carotenoids and detection of apocarotenoid volatiles was due to the action of the exogenous *AtCCD4* gene, transcript levels of three rice endogenous CCD genes (*OsCCD1*, *4a*, and *4b*) were examined by qRT-PCR in the two independent *AtCCD4*-transgenic rice lines T-1 and T-5 (Fig. 5). Two rice *CCD* genes, *OsCCD1* and *OsCCD4a*, showed no changes in their expression but the *OsCCD4b* gene displayed lower levels with 70 % lower expression than in NT control plants. These results strongly suggest that apocarotenoid production from the carotenoid cleavage activity of *AtCCD4* transgenic rice did not result from the action of the three endogenous rice *CCD* genes—*OsCCD1*, *4a*, and *4b*—but from the exogenous *AtCCD4* gene. RNA levels of another three endogenous rice CCD genes—*OsCCD7*, *8a*, and *8b*— were also analyzed and none of them was increased compared to NT control plants (Supplementary Table S2 and Fig. S2.).

Discussion

Two aspects of plant CCD enzymes have been the subject of research interest. The first area involves the tailoring of carotenoids into biologically functional apocarotenoids (in

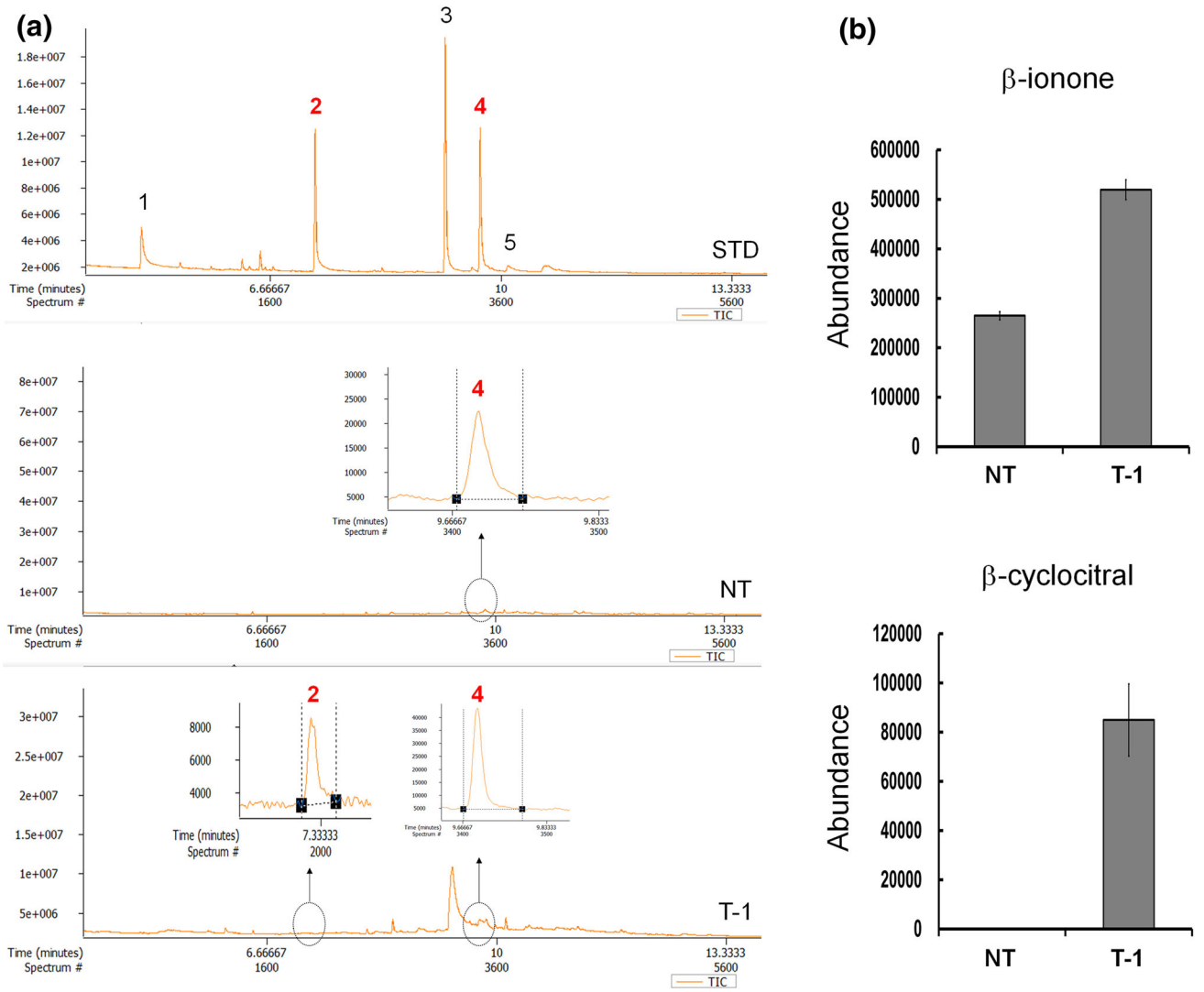

Fig. 4 Total ion chromatogram (**a**) and levels (**b**) of carotenoid cleavage products analyzed by GC–TOF–MS. STD, standard compounds including five carotenoid cleavage products (*1* 6-methyl 5-hepten-2-one, *2* β-cyclocitral, *3* α-ionone, *4* β-ionone, *5* pseudoionone), *NT* non-transgenic control rice plants, *T-1* homozygous T_4 plants of *AtCCD4* transgenic rice line 1. Two carotenoid cleavage products that varied between NT and the *AtCCD4* transgenic plants are indicated in *red* on the chromatograms

other words, CCPs), including hormones, flavors, and/or defense compounds and pigments. The other focus involves altering the carotenoid content to enhance the nutritional value of food crops and to change flower colors in horticultural crops (Bouvier et al. 2005; Auldridge et al. 2006a; Ilg et al. 2010; Walter et al. 2010; Gonzalez-Jorge et al. 2013). In the former group, the types of apocarotenoid products are highly dependent on the enzymatic action of the CCD family and the specific position of the double bonds cleaved on the carotenoids.

NCED families cleave at the 11–12 double bonds of 9-cis-epoxycarotenoid substrates (C_{40}) for abscisic acid biosynthesis (Walter and Strack 2011). CCD7 cleaves β-carotene (C_{40}) at the 9′–10′ position (not 9–10/9′–10′) to form a β-ionone (C_{13}) and a β-apo-10′-carotenal (C_{27}). The subsequent action of CCD8 cleaves this C_{27} apocarotenoid at the 13–14 position into a dialdehyde (C_9) and a β-apo-13-carotenone (C_{18}), which is an initial compound for strigolactone biosynthesis (Bouvier et al. 2005; Gomez-Roldan et al. 2008). Another CCD1 has been reported to cleave at diverse positions—5–6 (5′–6′), 7–8 (7′–8′), and 9–10 (9′–10′)—but preferentially at the 9–10 (9′–10′) position of cyclic and acyclic carotenoids to synthesize the C_{13} apocarotenoid volatiles of β-ionone and α-ionone in many plants (Schwartz et al. 2001; Simkin et al. 2004a, b; Baldermann et al. 2010; Walter and Strack 2011). In most CCD1 studies, the C_{13} CCP compounds were detected through gene expression in *E. coli* systems. Accordingly, the enhanced *in planta* β-ionone level has only been observed in *AtCCD1* transgenic *Arabidopsis* plants (Wei

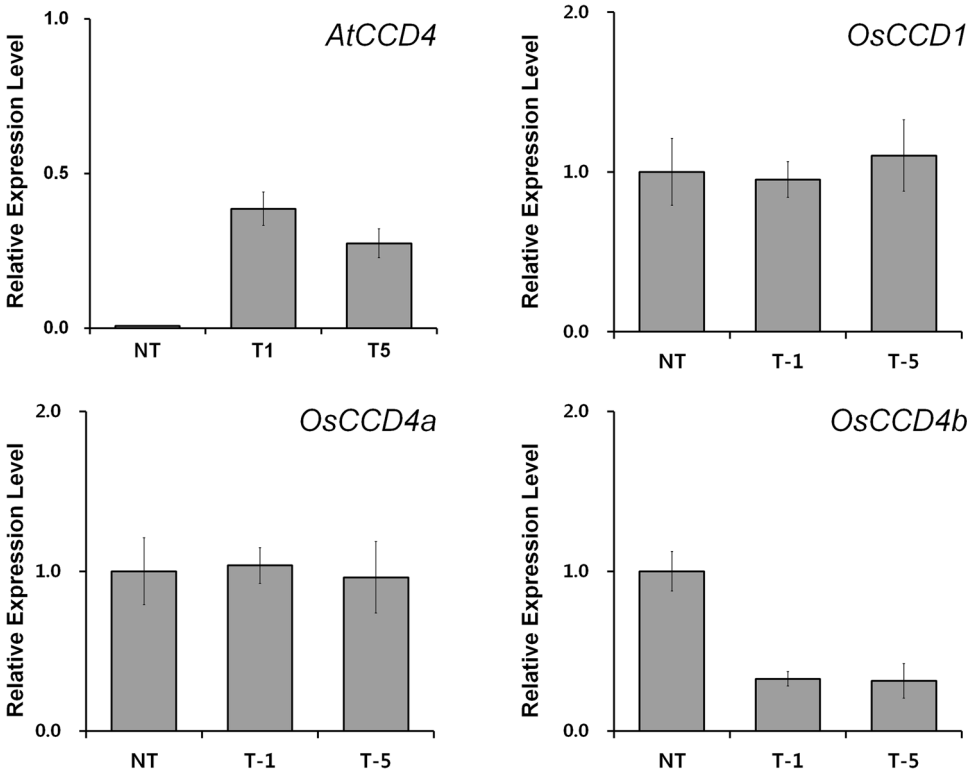

Fig. 5 Quantitative RT-PCR to compare the expression levels of three endogenous rice CCD genes (*OsCCD1*, *4a*, and *4b*) in two independent lines (T-1 and T-5) of *AtCCD4*-transgenic rice plants. The rice ubiquitin 5 gene (*OsUBQ5*) was used for normalization of the amounts of leaf RNA. NT means non-transgenic rice plants. Each value was the mean of three biological replicates

et al. 2011). In the case of CCD4 s, their carotenoid cleavage activities have also only been tested in *E. coli* systems, and not *in planta*. Among the six *CCD4* genes (*CsCCD4*, *AtCCD4*, *MdCCD4*, *CmCCD4a*, *RdCCD4*, and *OfCCD4*), three—*CsCCD4*, *MdCCD4*, and *CmCCD4a*—break a β-carotene and two—*AtCCD4* and *RdCCD*—lead to the degradation of an 8′-apo-β-caroten-8′-al (C_{30}) at the same 9–10 (9′–10′) position to produce a β-ionone (Rubio et al. 2008; Huang et al. 2009). Recently, enzymatic function of potato CCD4 (*StCCD4*) leading to β-ionone by catalyzing the cleavage of all-*trans*-β-carotene at the C9′–C10′ double bond was confirmed in *E.coli* (Bruno et al. 2015).

Thus, we examined the cleavage activity of AtCCD4 in *AtCCD4* transgenic rice plants, not in *E. coli*. Two independent single-copy rice lines (T-1 and T-5) were selected through TaqMan PCR and Southern blot analysis (Fig. 1). Overexpression of the *AtCCD4* transgene was confirmed by semiquantitative RT-PCR and qRT-PCR (Fig. 2). The total amount of carotenoids in the *AtCCD4* transgenic rice plant was 26 % lower than that in the NT plant. Four carotenoids—β-carotene, lutein, α-carotene, and violaxanthin—showed reduced levels at 74, 72, 51, and 78 % compared with the NT plant, respectively (Fig. 3 and Table S1). Given the small amounts of α-carotene and violaxanthin in rice leaves, we speculated that β-carotene and lutein might be the main substrates for direct and/or indirect exogenous AtCCD4 action.

To examine whether these decreases in the carotenoid levels were associated with the formation of CCPs by AtCCD4 enzymatic action, a GC-TOF–MS experiment was performed for targeted analysis using five CCP standards (α-ionone, β-ionone, pseudoionone, MHO, and β-cyclocitral). *AtCCD4* transgenic rice showed a two-fold higher level of β-ionone than NT plants and *de novo* production of β-cyclocitral (Fig. 4). Collectively, from carotenoid and apocarotenoid analyses, AtCCD4 could use β-carotene as cleavage substrates to generate β-ionone (C_{13}), and β-cyclocitral (C_{10}) as cleaved products. We cautiously suggest from this that AtCCD4 might be involved in cleavage actions at two positions of β-carotene: 9–10 (9′–10′) bonds for β-ionone and 7–8 (7′–8′)7–8 (7′–8′) for β-cyclocitral production.

Interestingly, β-ionone has a key role in attracting pollinators as fruit flavors and floral scents in tomato, grape, petunia, and *Osmanthus fragrans* Lour. (Simkin et al. 2004a; Simkin et al. 2004b; Mathieu et al. 2005; Baldermann et al. 2010). In addition, it endows *Arabidopsis* with insect resistance and inhibits the blue mold pathogen in tobacco, suggesting that CCPs are one of the convergent evolutionary ways for chemical defense in plants (Bouvier et al. 2005; Wei et al. 2011). As well as these biotic stresses, β-ionone and β-cyclocitral compounds are involved in responses to abiotic stresses, including extreme temperature and high levels of irradiation and ultraviolet (Bouvier et al. 2005; Rubio et al. 2008). Therefore,

AtCCD4 transgenic rice biofortified with two apocarotenoids (β-ionone and β-cyclocitral) could have value as biotic/abiotic stress-resistant crops with enhanced flavor. These agronomical benefits of diverse apocarotenoids will heighten expectations for the use of plant CCDs for future crop engineering and as promising targets for increasing the total amounts of provitamin A carotenoids. Thus, both the upregulation and downregulation of plant CCDs might be useful for crop biotechnology development.

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