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Acyclic carotenoid and cyclic apocarotenoid cleavage by an orthologue of lignostilbene-α,β-dioxygenase in *Rhodopseudomonas palustris*

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Carotenoid cleavage oxygenases catalyse formation of apocarotenoids and the precursors of phytohormones, abscisic acid and strigolactones through oxidative cleavage at specific double bonds of carotenoids. A gene encoding a presumed bacterial oxygenase homologous to lignostilbene-α,β-dioxygenases has been found in the genome of *Rhodopseudomonas palustris*. By analysing apocarotenoids in recombinant *Escherichia coli* strains, it was found that the presumed oxygenase catalyses the 15,15′ double bond cleavage of lycopene and neurosporene. Cell lysate containing the recombinant protein cleaved all-*trans*-β-apo-8′-carotenal at the 15,15′ double bond into retinal and apo-8′,15′-apocarotene-dial. These data demonstrate for the first time that the orthologue of lignostilbene-α,β-dioxygenase found in the carotenogenic phototrophic bacterium has the 15,15′ double bond cleavage activity towards both the acyclic carotenoids and cyclic apocarotenoid.

Keywords: apocarotenoid/carotenoid/carotenoid cleavage/photosynthetic bacterium/retinal.

Abbreviations: CCDs, carotenoid cleavage dioxygenases; CCO, carotenoid cleavage oxigenase; EI, electron impact ionization; HPLC, high-performance liquid chromatography; LSDs, lignostilbene-α, β-dioxygenases; ORF, open reading frame; PCR, polymerase chain reaction.

Carotenoid cleavage oxygenases (CCOs) catalyse formation of apocarotenoids and the precursors of phytohormones, abscisic acid and strigolactones through oxidative cleavage at internal C-C double bonds of carotenoids, which gives rise to the corresponding aldehydes and ketones (*1*). Several CCOs with different cleavage sites have been found in higher plants such as *Arabidopsis thaliana* (*2*), *Zea mays* (*3*) and *Osmanthus fragrans* (*4*) as well as in animals such as human, mouse and chicken (*5*, *6*). Compared with their variety found in higher plant species, however, prokaryotic CCO orthologues have been reported only in several species of cyanobacteria (*7*), *Mycobacterium tuberculosis* (*8*), *Novosphingobium aromaticivorans*, *Bradyrhizobium* sp. (*9*), *Pseudomonas paucimobilis* (*10*, *11*), *Sphingopyxis alaskensis*, *Plesiocystis pacifica* (*12*) and uncultured marine bacterium (*13*) even though various bacterial species are known to synthesize carotenoids (*14*).

It has been reported in cyanobacterium *Nostoc* sp. PCC 7120 that carotenoid cleavage dioxygenase NosCCD (NSC1) and apocarotenoid oxygenase NosACO (NSC2) cleave β,β-carotene at the 9,10 and 15,15′ positions, respectively (*15*). The third enzyme (NSC3) does not cleave full-length carotenoids but cleaves an apocarotenoid β-apo-8′-carotenal at the 9,10 position. Thus, the diversity of carotenoid cleavage activities has been identified in one cyanobacterium. In addition to the existence of paralogues, characterization of the products cleaved by NSC1 has revealed that the dioxygenase has two different cleavage patterns (*16*).

Carotenoid cleavage patterns and substrate specificities in cyanobacterial CCDs are closely related with those in several orthologues of higher plants, whereas homologues of carotenoid oxygenase, designated as lignostilbene-α,β-dioxygenases (LSDs), have been reported in non-carotenogenic bacteria (*9*–*11*) (Fig. 1). It has been shown that the LSD homologues in *N. aromaticivorans* and *Bradyrhizobium japonicum* cleave neither carotenoids nor apocarotenoids. Although several orthologues of cyanobacterial CCOs have been reported and characterized, only one orthologue, which belongs to the group of LSDs and is highly homologous to that in *B. japonicum* (*9*), has been found in non-cyanobacterial phototrophic bacterium *Rhodopseudomonas palustris*.

This study was performed to clarify whether or not the putative gene found in *R. palustris*, ORF *RPA1208*, encodes functional oxygenase and, if so, whether it cleaves both carotenoids and apocarotenoids. To confirm this, metabolites in cells of lycopene- or neurosporene-synthesizing recombinant *Escherichia coli* transformed with *RPA1208* and *in vitro* cleavage products of all-*trans*-β-apo-8′-carotenal by the recombinant enzyme were analysed.

Materials and Methods

Bacterial strains and growth conditions

Rhodopseudomonas palustris CGA009 (*17*) was grown in modified Okamoto medium at 30°C (*18*). *Pantoea agglomerans* (*Erwinia*

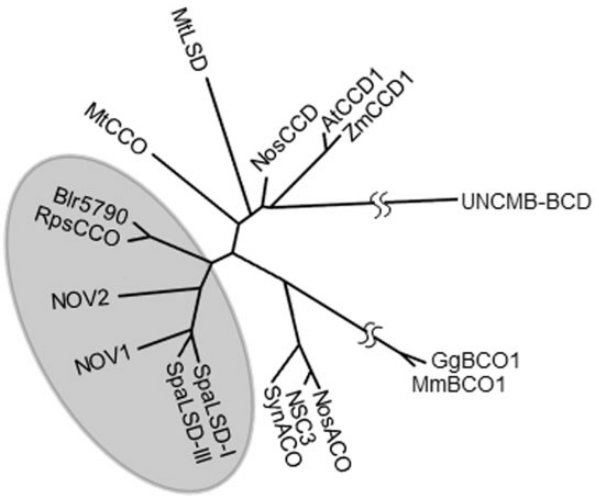

Fig. 1 Phylogenic tree composed of bacterial carotenoid oxygenases and their plant and animal representatives. *Rhodopseudomonas palustris* CGA009 RpsCCO (NP_946559); *Nostoc* sp. PCC 7120 NosCCD (NSC1) (NP_485149), NosACO (NSC2) (NP_488324) and NSC3 (NP_488935); *Synechocystis* sp. PCC 6803 SynACO (NP_441748); *N. aromaticivorans* DSM 12444 Lignostilbene oxygenase NOV1 (YP_496081) and NOV2 (YP_498079); *S. paucimobilis* Lignostilbene-α,β-dioxygenase isozyme I SpaLSD-I (AAC60447) and isozyme III SpaLSD-III (AAB35856); *B. japonicum* USDA 110 Blr5790 (NP_772430); *M. tuberculosis* H37Rv carotenoid cleavage oxygenase MtCCO (O06785) and putative Lignostilbene-α,β-dioxygenase MtLSD (NP_215428); uncultured marine bacterium 66A03 β-carotene 15,15′-dioxygenase UNCMB-BCD (Q4PNI0); *A. thaliana* AtCCD1 (NP_191911); *Z. mays* ZmCCD1 (NP_001105815); *Mus musculus* β-carotene dioxygenase 1 MmBCO1 (NP_067461); *Gallus gallus* β-carotene dioxygenase 1 GgBCO1 (NP_989966).

herbicola) NBRC12686 (National Institute of Technology and Evaluation, Kisarazu, Japan) was cultivated overnight in 5 ml Luria-Bertani (LB) medium at 30°C. *Escherichia coli* DH5α, JM109 and BL21(DE3) were purchased from Takara-bio (Shiga, Japan) and cultivated at 37°C. For construction of plasmids and cultivation of recombinant *E. coli* strains, antibiotics were added to LB medium at concentrations of 20 μg/ml for tetracycline, 30 μg/ml for kanamycin and 50 μg/ml for ampicillin. For production of apocarotenoids, *E. coli* cells were inoculated into 200 ml LB medium containing 2.5% (w/v) glycerol and 0.2% arabinose and cultured in the presence of antibiotics for 24 h at 30°C with gentle shaking.

Construction and introduction of plasmids

A plasmid encoding genes related to lycopene biosynthesis was constructed as follows. *Erwinia uredovora crtE* and *crtB* were amplified with PCR using a DNA template pACCRT-EB (*19*) and primers crtES and crtEA for *crtE* or primers crtBS and crtBA for *crtB* (Supplementary Table S1). The PCR fragments were cloned into pGEM-T easy vector (Promega, Tokyo, Japan), resulting in pGEMcrtE and pGEMcrtB. *Pantoea agglomerans crtI* was amplified with PCR using the bacterial total DNA extracted with a DNeasy tissue kit (Qiagen, Tokyo, Japan) and primers crtIS and crtIA (Supplementary Table S1). The PCR fragment was cloned into pGEM-T easy vector, resulting in pGEMcrtI. The three plasmids, pGEMcrtE, pGEMcrtB and pGEMcrtI, were digested with endonucleases and generated DNA fragments were ligated in an order of crtEIB. Finally, a crtEIB fragment was amplified with PCR using primers crtEIBS and crtEIBA (Supplementary Table S1), digested with XbaI and introduced into the XbaI site of pHY300PLK vector (Takara Bio, Shiga, Japan), resulting in pHYcrtEIB. For construction of a set of neurosporene- and lycopene-producing *E. coli* strains, pACCRT-EBI$_{Rc}$ and pACCRT-EBI$_{Eu}$ (*20*) were introduced, respectively, instead of pHYcrtEIB.

A plasmid including *R. palustris* CGA009 open reading frame (ORF) *RPA1208* was constructed as follows. The ORF together with its native Shine–Dalgarno sequence was amplified with polymerase chain reaction (PCR) using the bacterial total DNA extracted with a DNeasy tissue kit (Qiagen, Tokyo, Japan) and primers ccoS and ccoA (Supplementary Table S1). The PCR fragment was cloned into pGEM-T easy vector, resulting in pGEMRpscco. A DNA fragment including the ORF was generated with endonuclease digestion and ligated to the KpnI and XbaI sites of pMG103 (*21*), resulting in pMGRpscco.

Cells of JM109 were transformed with pHYcrtEIB, pACCRT-EBI$_{Rc}$ or pACCRT-EBI$_{Eu}$ by heat shock to isolate carotenoid-producing *E. coli* JM109 strains. An empty vector pMG103 or pMGRpscco was introduced into cells of JM109 (pHYcrtEIB), JM109 (pACCRT-EBI$_{Rc}$), JM109 (pACCRT-EBI$_{Eu}$) and BL21 (DE3) (Merck, Tokyo, Japan) by electroporation using Gene pulsar (Bio-Rad Laboratories, Hercules, CA, USA) with settings at 12.5 kV/cm field strength, 25 μF capacitance and 300 Ω electrical resistance.

Phylogenetic analysis

A phylogenetic tree based on amino acid sequences of CCO homologues was constructed using ClastalW at the DDBJ (DNA Databank of Japan, Shizuoka, Japan) web site. The amino acid sequences were obtained using the accession numbers indicated below from the NCBI (National Center for Biotechnology Information) web site.

Extraction and analyses of apocarotenoids

Escherichia coli cells were harvested from 200 ml culture by centrifugation, resuspended with 2 ml distilled water and transferred to a screw-capped glass tube. Cell suspension was centrifuged at 450×*g* for 10 min and the supernatant was removed from the glass tube. Cells were resuspended in 2 ml methanol to extract apocarotenoids and stirred on a Vortex mixer for 5 min. Then, the cell suspension was incubated for 10 min at 55°C and again stirred by a Vortex mixer for 5 min. The cell suspension was centrifuged at 450×*g* for 10 min and the supernatant was collected. The extraction with methanol was repeated twice.

The methanol fractions were combined and filtered to subject to a high-performance liquid chromatography (HPLC) system composed of a binary pump 1525 and an autosampler 717plus (both from Waters, Milford, MA, USA) equipped with a μBondapak C_{18} column (3.9 by 300 mm; particle size, 10 μm; pore size, 125 Å) (Waters, Milford, MA, USA). Apocarotenoids in 200 μl of the methanol fractions were analysed and fractionated with a mobile phase composed of methanol:acetonitrile:water (19:19:2, v/v/v) at a flow rate of 1.0 ml/min. Absorbance was monitored with a photodiode array detector 2996 (Waters, Milford, MA, USA) within the range 230–800 nm.

One microlitre of HPLC fraction was subjected to a gas chromatograph Focus GC coupled with a quadrapole mass spectrometer DSQII (GC–MS) (Thermo Fisher Scientific, Yokohama, Japan). The column oven was programmed from 63°C to 250°C at a heating rate of 10°C/min. The injection and ion source temperatures were set at 220°C and 250°C, respectively. High purity helium was used as a carrier gas at a flow-rate of 1.2 ml/min and apocarotenoids were separated through an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Hewlett-Packard, Atlanta, GA, USA). Mass spectra were recorded in the electron impact ionization (EI) mode.

In vitro cleavage assay

The carotenoid-cleaving plasmid pMGRpscco or empty vector pMG103 was introduced into *E. coli* BL21 (DE3). Transformants were inoculated into 5 ml medium and cultured for 15 h at 30°C with gentle shaking. Then, 2 ml of the pre-cultures were inoculated into 200 ml medium, and cultured for 15 h at 20°C with gentle shaking. The cells were collected by centrifugation at 4°C, washed once with phosphate buffer (0.1 M potassium phosphate pH 7.0) and resuspended into 10 ml phosphate buffer. The cells in a glass vial placed on ice were disrupted for 5 min with a 20 kHz ultrasonic disruptor equipped with a microchip probe (UD-201, Tomy, Tokyo, Japan). Sonication with output power of 40 W was repeated four times with 5 min interval. Cell lysate was obtained after removal of cell debris by centrifugation at 16,000×*g* for 15 min at 4°C.

In vitro cleavage assay was performed with all-*trans*-β-apo-8′-carotenal (Sigma-Aldrich, Tokyo, Japan) as a substrate. The apocarotenoid was dissolved in 5 ml ethanol containing 0.1% (w/v) Triton X-100 at a concentration of 0.06 mg/ml. After evaporation of ethanol using rotary evaporator, 3 ml of the cell lysate was added immediately. Then, the mixture was transferred into a glass vial and incubated in the dark at 30°C with gentle shaking. After a 24 h incubation,

apocarotenoids were extracted from 500 µl of the mixture with 500 µl *n*-hexane-diethyl ether (1:4, v/v). One microlitre of the organic phase was subjected to GC–MS analysis as described above.

Results and Discussion

Phylogenetic analysis for a CCO homologue in R. palustris

An ORF encoding putative carotenoid cleavage oxygenase *RPA1208* has been found in the genome of *R. palustris*. When the amino acid sequence of the putative CCO (RpsCCO) was compared with bacterial homologues experimentally analysed previously, the highest identity (73%) was obtained against a putative lignostilbene-α,β-dioxygenase of *B. japonicum* USDA 110 (Blr5790). The phylogenetic tree constructed from amino acid sequences of bacterial, animal, bird and plant CCO homologues showed a close phylogenetic relationship between RpsCCO and Blr5790, both of which were included in a group of bacterial lignostilbene-α,β-dioxygenases (Fig. 1).

Apocarotenoid formation in recombinant E. coli *strains*

When pMGRpscco encoding RpsCCO was introduced to lycopene-producing *E. coli* JM109 (pHYcrtEIB), the cells were bleached after cultivation (Fig. 2A). To confirm cleavage of lycopene in *E. coli* JM109 (pHYcrtEIB, pMGRpscco), apocarotenoid formation in the cells was analysed with HPLC. A peak with the absorption maximum of 395 nm was found in the chromatogram of cell extract monitored at a wavelength of 400 nm (Fig. 2B, peak *a*). Peak *a* was not found when the cell extract of JM109 (pHYcrtEIB, pMG103) was loaded into HPLC. When a fraction containing peak *a* was collected and subjected to GC–MS, molecular ion at $m/z = 284$ was detected in the mass spectrum (Fig. 3). From the chemical structure of lycopene, the absorption maximum and the m/z of molecular ion, peak *a* was identified as γ-retinal (MW 284), which was an apocarotenoid produced as a result of the 15,15′ cleavage of lycopene.

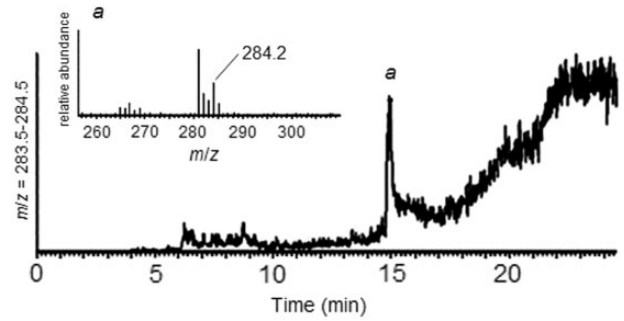

Fig. 3 GC–MS analysis of apocarotenoid produced after lycopene cleavage by RpsCCO in *E. coli* cells. The chromatogram was obtained with an apocarotenoid fraction purified by HPLC from the extract of cells harbouring pMGRpscco. The inset indicates mass spectrum of peak *a*.

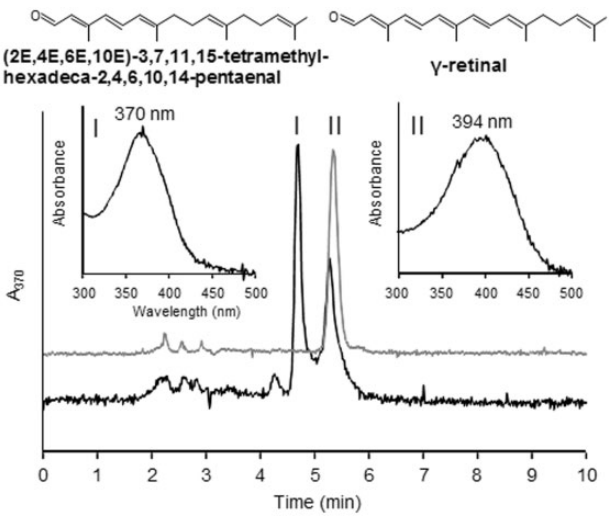

Fig. 4 Apocarotenoid formation from neurosporene in *E. coli* cells. The chromatograms were obtained with extracts from neurosporene-producing cells harbouring both pACCRT-EBI$_{Rc}$ and pMGRpscco (black) or lycopene-producing cells harbouring both pACCRT-EBI$_{Eu}$ and pMGRpscco (grey). The insets indicate absorption spectra of peaks I and II.

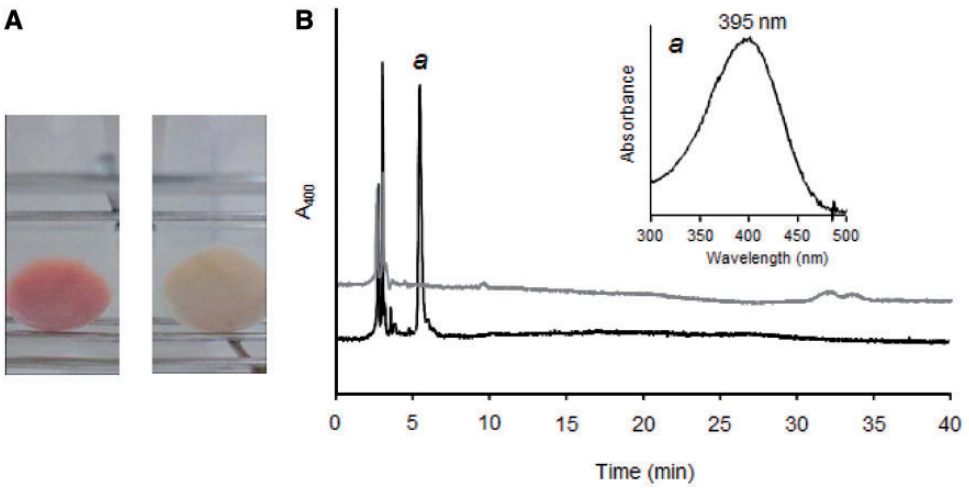

Fig. 2 Lycopene cleavage and apocarotenoid formation by a putative carotenoid cleavage oxygenase (RpsCCO). *Escherichia coli* cells accumulating lycopene (Left) was bleached when pMGRpscco was introduced (Right) (A). HPLC analysis of apocarotenoid extracted from *E. coli* cells producing RpsCCO (B). Analytes were extracted from cells harbouring pMGRpscco (black) or pMG103 (grey). The inset indicates absorption spectrum of peak *a*.

Using JM109 (pACCRT-EBI$_{Rc}$, pMGRpscco) harbouring the neurosporene-synthesizing pathway, apocarotenoid formation from neurosporene catalysed by RpsCCO was examined (Fig. 4). As observed in JM109 (pHYcrtEIB, pMGRpscco) harbouring the lycopene-synthesizing pathway, a peak with the 394 nm absorption maximum (peak II) was found in lycopene-cleaving JM109 (pACCRT-EBI$_{Eu}$, pMGRpscco) cells. From the retention time and 394 nm absorption maximum of peak II, γ-retinal formation from lycopene cleavage catalysed by RpsCCO was also suggested in the recombinant *E. coli* cells harbouring the different lycopene-producing plasmid. When neurosporene was produced instead of lycopene in JM109 (pACCRT-EBI$_{Rc}$, pMGRpscco), another peak with the 370 nm absorption maximum (peak I) emerged in addition to peak II. The shift of the absorption maximum to the short-wavelength (370 nm) suggested peak I as (2E,4E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,4,6,10,14-pentaenal (Fig. 4), which is one of the 15,15′ cleavage products of neurosporene and has the smaller number of conjugated double bonds as a chromophore.

In vitro cleavage assay

All-*trans*-β-apo-8′-carotenal was mixed with cell lysates obtained from *E. coli* BL21 (DE3) harbouring pMGRpscco. After a 2 h incubation, the colour of reaction mixture changed from red to orange (Fig. 5A). Addition of cell extract of BL21 (DE3) harbouring an empty vector pMG103 did not cause colour change of reaction mixture after incubation (Supplementary Fig. S1). Therefore, it was suggested that *cco* gene was expressed to produce recombinant RpsCCO protein in BL21 (DE3) harbouring pMGRpscco. When 1.0 mM Fe^{2+} was added to the reaction mixture, the orange or yellow discolouration was accelerated (Supplementary Fig. S1), indicating that RpsCCO requires Fe^{2+} as a co-factor and possesses the common feature preserved within carotenoid oxygenase family (*5*).

Then, the reaction products were analysed by GC–MS. Peaks 1 and 2 were detected in the chromatograms monitoring ions of $m/z = 284$ (Fig. 5B) and $m/z = 164$ (Fig. 5C), respectively, when the cell lysate of BL21 (DE3, pMGRpscco) was mixed with β-apo-8′-carotenal. These two peaks were not detected when the reaction mixture with the cell lysate of BL21 (DE3, pMG103) was subjected to GC–MS analysis. From the mass spectra of peaks 1 and 2, it was concluded that α-retinal (MW 284) and apo-8′,15′-apocarotene-dial (MW 164) were formed after cleavage of β-apo-8′-carotenal catalysed by recombinant RpsCCO protein.

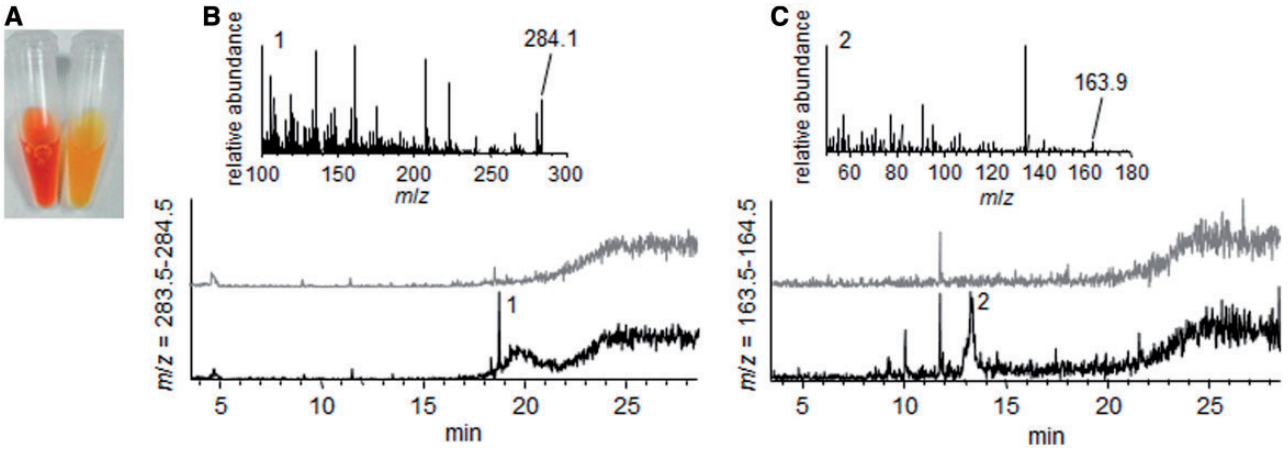

Fig. 5 *In vitro* cleavage of apocarotenoid by RpsCCO. Reaction mixtures before and 2 h after the addition of enzyme (A). GC–MS analysis of extracts from reaction mixtures of all-*trans*-β-apo-8′-carotenal and cell lysate of BL21(DE3) harbouring pMGRpscco (Black) or pMG103 (Grey). Chromatograms monitoring ions of $m/z = 283.5$–284.5 (A) and $m/z = 163.5$–164.5 (B). Insets show mass spectra of peaks 1 and 2.

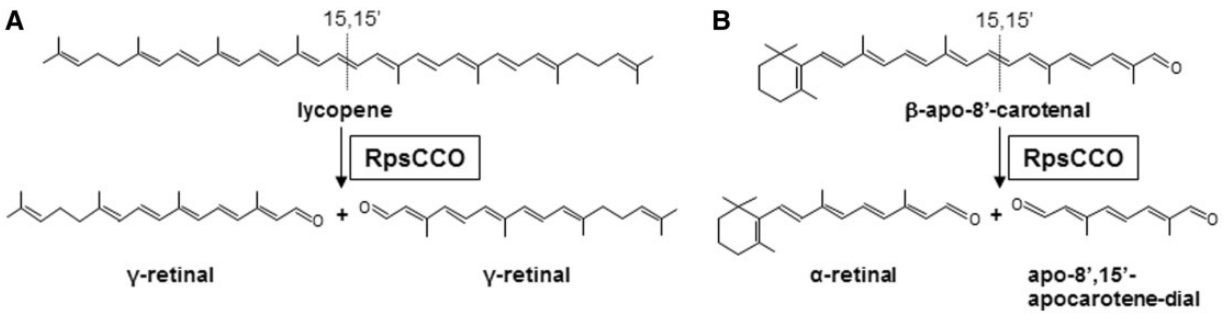

Fig. 6 Proposed cleavage sites of lycopene (A) and all-*trans*-β-apo-8′-carotenal (B) by RpsCCO. Dashed lines indicate cleavage at the 15,15′ double bond.

Carotenoid and apocaroteoid cleavage by RpsCCO

In vivo and *in vitro* cleavage assays performed in this study revealed that RpsCCO cleaves 15,15′ double bond of the acyclic carotenoids, lycopene and neurosporene, and the cyclic apocarotenoid, all-*trans*-β-apo-8′-carotenal (Fig. 6). However, *in vitro* cleavage activity towards β-carotene could not be detected even though *in vitro* cleavage activity towards lycopene to form γ-retinal was also found as observed in *in vivo* cleavage assay using JM109 (pHYcrtEIB, pMGRpscco) and JM109 (pACCRT-EBI$_{Eu}$, pMGRpscco) (Data not shown). The phylogenetic analysis among the CCO homologues indicates that RpsCCO belongs to the group of alphaproteobacterial lignostilbene-α,β-dioxygenases and it is highly homologous to *B. japonicum* USDA 110 (Blr5790). However, the putative lignostilbene-α,β-dioxygenases of *Bradyrhizobium* sp. (BRA-J and BRA-S) cleave neither stilbene, carotenoid nor apocarotenoid (*9*). Among the homologues of lignostilbene-α,β-dioxygenases found in alphaproteobacteria, the stilbene-cleaving oxygenases of *N. aromaticivorans* DSM 12444 (NOV1 and NOV2) do not cleave carotenoids or apocarotenoids (*9*). The non-cyanobacterial carotenoid oxygenases that have specificities to carotenoid and apocarotenoid have been found in *M. tuberculosis* H37Rv (*8*), *S. alaskensis* RB2256 and *P. pacifica* SIR-1 (*12*). Phylogenetic analysis of CCO homologues revealed that these non-cyanobacterial carotenoid oxygenases that cleave carotenoids and apocarotenoids are not closely related to the alphaproteobacterial lignostilbene-α,β-dioxygenase homologues, even though *S. alaskensis* is a member of *Alphaproteobacteria*. Therefore, RpsCCO is the first carotenoid cleavage oxygenase in the group of bacterial lignostilbene-α,β-dioxygenase homologues cleaving the 15,15′ double bond of carotenoids and apocarotenoids to form retinals.

Supplementary Data

Supplementary Data are available at *JB* Online.

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