

New Insight into the Cleavage Reaction of *Nostoc* sp. Strain PCC 7120 Carotenoid Cleavage Dioxygenase in Natural and Nonnatural Carotenoids

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Carotenoid cleavage dioxygenases (CCDs) are enzymes that catalyze the oxidative cleavage of carotenoids at a specific double bond to generate apocarotenoids. In this study, we investigated the activity and substrate preferences of NSC3, a CCD of *Nostoc* sp. strain PCC 7120, *in vivo* and *in vitro* using natural and nonnatural carotenoid structures. NSC3 cleaved β -apo-8'-carotenal at 3 positions, C-13=C-14, C-15=C-15', and C-13'=C-14', revealing a unique cleavage pattern. NSC3 cleaves the natural structure of carotenoids 4,4'-diaponeurosporene, 4,4'-diaponeurosporen-4'-al, 4,4'-diaponeurosporen-4'-oic acid, 4,4'-diapotorulene, and 4,4'-diapotorulen-4'-al to generate novel cleavage products (apo-14'-diaponeurosporenal, apo-13'-diaponeurosporenal, apo-10'-diaponeurosporenal, apo-14'-diapotorulenal, and apo-10'-diapotorulenal, respectively). The study of carotenoids with natural or nonnatural structures produced by using synthetic modules could provide information valuable for understanding the cleavage reactions or substrate preferences of other CCDs *in vivo* and *in vitro*.

Carotenoids belong to a class of isoprenoid derivatives found in many organisms, including photosynthetic and nonphotosynthetic bacteria (1). They have diverse biological functions, such as involvement in coloration, antioxidation activity, light harvesting, energy transfer, membrane fluidity regulation, and the quenching of singlet oxygen or peroxy radicals (2–4). To date, more than 700 carotenoids have been identified and classified as C₃₀, C₄₀, and C₅₀ carotenoids, depending on the number of carbons in their carotene backbones (5).

Animals do not biosynthesize carotenoids *de novo* and thus obtain carotenoids mainly from dietary fruits or vegetables. Most carotenoids consumed (including xanthines) are oxidatively cleaved by carotenoid cleavage dioxygenases (CCDs), and the resulting cleavage compounds (for example, retinal) function as transcriptional system activators or cellular components (6).

CCDs are enzymes that catalyze the oxidative cleavage of carotenoids; they are found in animals, plants, photosynthetic bacteria, algae, and cyanobacteria. The cleavage of carotenoids results in the formation of structurally diverse apocarotenals that carry out biological functions, including their general roles as antioxidants, agents of volatile aromas and flavors, provitamin A, and regulators of retinoids (7). The physiological functions of apocarotenoids in plants as precursors of phytohormones, such as abscisic acid and strigolactone, have been reported recently (8–10). For example, strigolactone, which triggers seed germination in plants, is derived from C₁₈ β -apo-13-carotenone, which is produced when CCD8 cleaves C₂₇ β -apo-10'-carotenal (9). The elucidation of novel aspects of potential health-promoting mechanisms has drawn increasing attention to the effects of diverse apocarotenoid structures (6). Bacterial CCDs, except for those of cyanobacteria such as *Synechocystis* and *Nostoc*, as well as *Anabaena* spp., have not been well studied yet. Recently, CCDs have been identified in several proteobacteria: *Novosphingobium aromaticivorans*, *Sphingopyxis alaskensis*, and *Plesiocystis pacifica* (11, 12). *Mycobacterium tuberculosis* contains CCDs in its genome, although it cannot biosynthesize carotenoids (13).

In *Nostoc* sp. strain PCC 7120, a representative carotenogenic cyanobacterium, retinal was first noted as a chromophore of the light-activated photoreceptor rhodopsin (14). Three CCDs, NSC1 (*Nostoc* CCD [NosCCD]; GenBank accession no. [NP_485149](#)), NSC2 (*Nostoc* apocarotenal cleavage oxygenase [NosACO]; GenBank accession no. [NP_488324](#)), and NSC3 (*Nostoc* dioxygenase 2 [NosDiox2]; GenBank accession no. [NP_488935](#)) (Fig. 1A) have been identified in *Nostoc* sp. PCC 7120 (15–17). Soluble NSC1, which is encoded by *all1106*, cleaves *in vitro* C-9=C-10 and C-9'=C-10' double bonds in bicyclic carotenoids, including β -carotene, and C-9=C-10 and C-7'=C-8' double bonds in monocyclic carotenoids, including myxoxanthophyll. NSC2, which is encoded by *all4284*, cleaves monocyclic or acyclic (linear) carotenoids at C-15=C-15' double bonds to generate retinal *in vitro* but barely cleaves β -carotene *in vivo*. NSC3, which is encoded by *all4895*, cleaves β -apo-8'-carotenal at C-9=C-10 double bonds *in vitro*, but no detailed biochemical activity of NSC3 *in vivo* or *in vitro* has been reported (16, 17). Furthermore, the preferred *in vivo* and *in vitro* substrates have not been determined, owing mainly to the unavailability of carotenoids with various structures.

Synthetic biological approaches have been widely used for the redesign and reconstruction of biosynthetic pathways in heterologous hosts (18, 19). The functional, modular expression of redesigned pathway enzymes can improve the productivity and yield of target molecules (20). Moreover, the modular expression of a pathway enzyme can be used as a tool for diversifying the struc-

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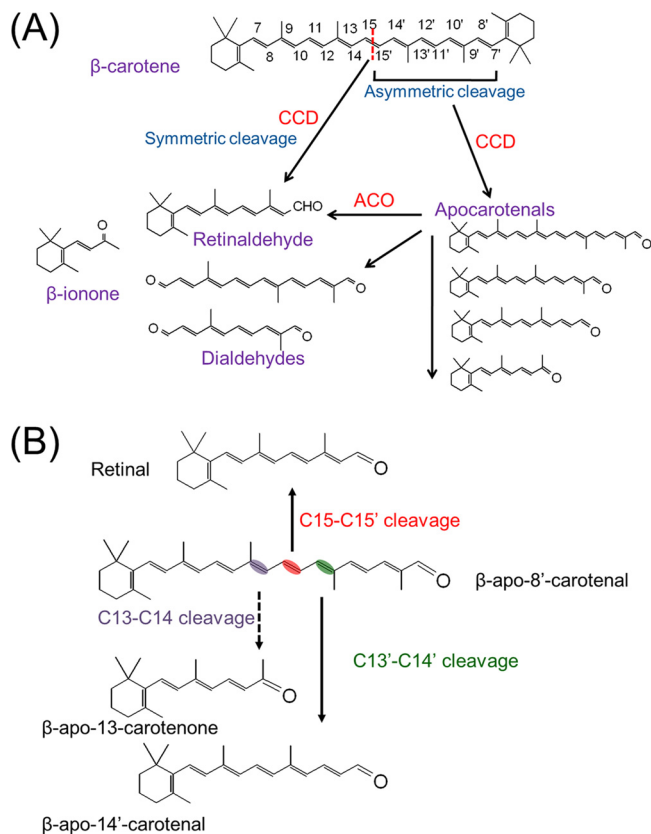


FIG 1 Selected cleavage patterns of β -carotene and β -apo-8'-carotenal. (A) Carotenoid cleavage reactions for various cleavage enzymes. CCD, carotenoid cleavage dioxxygenase; ACO, apocarotenal cleavage oxygenase. (B) Characterized cleavage sites on β -apo-8'-carotenal and resulting cleavage products catalyzed by NSC3 in this study.

tures of natural and nonnatural molecules, such as carotenoids, by using combinatorial biosynthesis in heterologous hosts (21). Therefore, carotenoids with natural or nonnatural structures, including natural or nonnatural C_{30} carotenoids, can provide new insight into the cleavage reactions or substrate preferences of CCDs, including NSC3, *in vivo* and *in vitro*.

Here we describe the activity and substrate preferences of NSC3 *in vitro* and *in vivo* with natural and nonnatural carotenoid structures generated using synthetic carotenoid modules in *Escherichia coli*. We reveal that NSC3 has unique cleavage activity (C-13=C-14, C-15=C-15', and C-13'=C-14') with respect to β -apo-8'-carotenal *in vitro* (Fig. 1B), as well as with other carotenoid structures *in vivo* and *in vitro*. In addition, we found that NSC3 uses natural (4,4'-diaponeurosporene, 4,4'-diaponeurosporen-4'-al, and 4,4'-diaponeurosporen-4'-oic acid) and non-natural (4,4'-diapotorulene and 4,4'-diapotorulen-4'-al) C_{30} carotenoids (not apocarotenoids) as substrates to generate structurally novel cleavage products. These novel apocarotenal structures might be bioactive candidates of biotechnological significance.

MATERIALS AND METHODS

Cloning and culture conditions. We amplified *all4895*, which encodes NSC3, from the genomic DNA of *Nostoc* sp. PCC 7120 by using PCR with a 5' primer that contained an XbaI restriction enzyme site followed by an

optimized Shine-Dalgarno sequence (underlined) and a start codon (boldface) (5'-AGGAGGATTACAAAATG-3') and a 3' primer that contained an EcoRI restriction enzyme site at its 5' end. The PCR product was purified with a PCR product purification kit (MacroGen, South Korea). It was then digested using XbaI and EcoRI (New England BioLabs) and was ligated into the corresponding restriction sites of plasmid pUCM (22). For the overexpression of His₆-tagged NSC3, the gene that encodes NSC3 was subcloned into the NdeI and XhoI sites of vector pET21a by using gene-specific primers that contained the corresponding restriction sites. For *in vivo* cleavage reaction studies, NSC3 was subcloned into the EcoRI and BamHI sites of pBBR1MCS-2, which can coexist with a plasmid that encodes carotenogenic gene modules. All of the cloning and *in vivo* analyses were carried out in *E. coli* strain JM109. *Escherichia coli* cultures were grown at 30°C in Luria-Bertani (LB) medium supplemented with an appropriate selective antibiotic (ampicillin [100 μ g/ml], chloramphenicol [50 μ g/ml], or kanamycin [30 μ g/ml]) as needed. All of the plasmids and strains used in this study are listed in Table 1.

Expression and purification of NSC3. *Escherichia coli* BL21(DE3) was used to express and purify recombinant His₆-tagged NSC3. To increase the expression of soluble NSC3, we cotransformed the pET21-NSC3 plasmid with a chaperone plasmid (pGro7 or pKJE7) carrying *groES-groEL* or *dnaK-dnaJ-grpE*, respectively (TaKaRa). The cotransformants were grown in 500 ml LB medium in a 2-liter flask containing 100 μ g/ml ampicillin at 30°C with shaking at 210 rpm. When the absorbance of a culture reached 0.6 at 600 nm, 1 mM isopropyl β -D-thiogalactopyranoside and 0.5 mg/ml L-arabinose were added to induce the expression of the NSC3 and chaperone proteins. After induction, the culture was incubated at 18°C for another 16 h. After centrifugation (4,000 rpm, 30 min, and 4°C), the cell pellets were washed twice with 1 $\times$ phosphate-buffered saline (PBS) buffer and were then resuspended in a phosphate buffer that contained 50 mM Na₂PO₄ at pH 7.0. Resuspended cells were sonicated on ice (20% power with pulsing [5 s on and 10 s off] for 5 min), and the cell debris was removed via centrifugation at 13,000 rpm for 30 min at 4°C.

The supernatant was concentrated using a Vivaspin 20 centrifugal filter (molecular weight cutoff, 30,000; Sartorius) and was applied to a HisTrap HP affinity chromatography column (GE Healthcare, Munich, Germany) equilibrated with 20 mM phosphate buffer (pH 8.0) containing 500 mM NaCl. The column was washed extensively with the same buffer, and the bound protein was eluted with a linear gradient between 20 and 500 mM imidazole (Sigma) at a flow rate of 1 ml/min. The purification step involving chromatography was performed using an ÄKTA fast protein liquid chromatography (FPLC) system (GE Amersham-Pharmacia). The fractions containing the recombinant protein were immediately pooled, concentrated in a Vivaspin 20 centrifugal filter (Sartorius), desalted with a PD10 desalting column (Amersham), and finally concentrated in 50 mM sodium phosphate buffer (pH 7.0). The proteins were quantified by the Bradford method, and homogeneity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Coomassie brilliant blue staining. The purified His₆-tagged NSC3 was concentrated to $\sim$ 2 mg/ml.

In vitro assays. *In vitro* reactions were performed using 5 μ g purified NSC3 in a 300- μ l reaction mixture consisting of 50 mM phosphate buffer (pH 7.0), 300 mM NaCl, 0.5 mM FeSO₄, and 10 mM dithiothreitol. A carotenoid substrate including β -apo-8'-carotenal was dissolved in *tert*-methyl ethyl ester containing Tween 40 (final concentration, 0.3% [vol/vol]) to form detergent micelles, and the solution was sonicated for 30 min using an ultrasonicator (Branson). The solvent was evaporated using an EZ-2 Plus centrifugal evaporator (Genevac, Gardiner, NY), and then the reaction buffer was added. The resulting mixture was used as a substrate solution. To examine the effect of pH on the activity of NSC3, we varied the pH between 5.0 and 9.0 using 50 mM sodium acetate buffer (pH 5.0), 50 mM sodium phosphate buffer (pH 6.0, 7.0, and 8.0), and 50 mM glycine-NaOH buffer (pH 9.0). Then 50 μ M β -apo-8'-carotenal and 5 μ g enzyme were used for reactions at 30°C for 30 min. The relative activity of

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
<i>Nostoc</i> sp. PCC 7120	C ₄₀ carotenoid pathway and rhodopsin; also known as <i>Anabaena</i> sp. PCC 7120	UTEX 2576
<i>Escherichia coli</i> SURE	<i>endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC e14⁻ Δ(mcrCB-hsdSMR-mrr)171 F'[proAB⁺ lac^qlacZΔM15 Tn10]</i>	Stratagene
JM109	<i>endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB⁺ Δ(lac-proAB) e14⁻ [F' traD36 proAB⁺ lac^qlacZΔM15] hsdR17(r_K⁻ m_K⁺)</i>	New England BioLabs
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 Sam7 nin5])</i>	
Plasmids		
pUCM	Cloning vector modified from pUC19; constitutive <i>lac</i> promoter; Ap ^r	22
pBBR1MCS-2	Cloning vector; SC101 origin; inducible <i>lac</i> promoter; Km ^r	23
pGro7	Chaperone plasmid inducibly expressing <i>groES-groEL</i> ; <i>araB</i> promoter; Cm ^r	TaKaRa
pKJE7	Chaperone plasmid inducibly expressing <i>dnaK-dnaJ-grpE</i> ; <i>araB</i> promoter; Cm ^r	TaKaRa
pUCM-NSC3	Constitutively expressed <i>all4895</i> gene of <i>Nostoc</i> sp. PCC 7120	This study
pBBR-NSC3	Inducibly expressed <i>all4895</i> gene of <i>Nostoc</i> sp. PCC 7120	This study
pET21-NSC3	Inducibly expressed His ₆ -tagged <i>all4895</i> gene of <i>Nostoc</i> sp. PCC 7120	This study
pUCM-Y _{IBL}	Constitutively expressed <i>crtYcYd</i> , gene of <i>Brevibacterium linens</i>	Unpublished
pUCM-aldH _{SA}	Constitutively expressed aldehyde dehydrogenase gene of <i>Staphylococcus aureus</i>	19
pBBR-aldH _{SA}	Inducibly expressed aldehyde dehydrogenase gene of <i>S. aureus</i>	19
pACM-M _{SA} -N _{SA}	Constitutively expressed <i>crtM</i> and <i>crtN</i> genes of <i>S. aureus</i> on pACYC184	19
pACM-M _{SA} -N _{SA} -P _{SA}	Constitutively expressed <i>crtM</i> , <i>crtN</i> , and <i>crtP</i> genes of <i>S. aureus</i>	19
pACM-M _{SA} -N _{YSA} -Y _{IBL}	Constitutively expressed <i>crtM</i> , <i>crtN</i> , and <i>crtYcYd</i> , genes of <i>S. aureus</i> and <i>B. linens</i>	Unpublished
pACM-E _{PAG} -B _{PAG} -I _{PAG}	Constitutively expressed <i>crtE</i> , <i>crtB</i> , and <i>crtI</i> genes from <i>Pantoea agglomerans</i> to produce lycopene	24
pACM-E _{PAG} -B _{PAG} -I _{PAG} -Y _{PAG}	Constitutively expressed <i>crtE</i> , <i>crtB</i> , <i>crtI</i> , and <i>crtY</i> genes from <i>P. agglomerans</i> to produce β-carotene	24
pACM-E _{PAG} -B _{PAG} -I _{PAG} -Y _{PAG} -Z _{PAG}	Constitutively expressed <i>crtE</i> , <i>crtB</i> , <i>crtI</i> , <i>crtY</i> , and <i>crtZ</i> genes from <i>P. agglomerans</i> to produce zeaxanthin	24
pAC-E-B-I ₁₄	Constitutively expressed <i>crtE</i> , <i>crtB</i> , and <i>crtI</i> ₁₄ genes of <i>Pantoea ananatis</i>	25
pAC-E-B-I ₁₄ -Y ₂	Constitutively expressed <i>crtE</i> , <i>crtB</i> , <i>crtI</i> ₁₄ , and <i>crtY</i> ₂ genes of <i>P. ananatis</i>	26

NSC3 was monitored in 50 mM sodium phosphate buffer (pH 7.0) for 30 min at various temperatures between 15°C and 45°C.

Enzyme kinetic analysis. Initial measurements were carried out at 30°C using a UV spectrophotometer (SpectraMax Plus 384; Molecular Devices). Retinal and β-apo-8'-carotenal were quantified by peak integration using high-performance liquid chromatography (HPLC) analysis with known amounts of standard materials. To determine the kinetic values, we varied the concentration of β-apo-8'-carotenal between 20 and 100 μM, with the purified NSC3 concentration fixed at 5 μg. These enzymatic reactions were performed at 30°C for 30 min for each substrate concentration. The reactions were terminated by the addition of 300 μl chloroform. Subsequently, the samples were centrifuged at 13,200 rpm for 1 min, and the chloroform phase was collected. The treated samples were analyzed by HPLC using a Zorbax Eclipse XDB C₁₈ column (inner diameter, 4.6 mm; length, 150 mm; particle size, 5 μm; Agilent Technologies, Santa Clara, CA). Conversion was measured photometrically for the corresponding substrate and product absorption maxima. Plotted data were fit to a Michaelis-Menten curve using SigmaPlot, version 12.0. Experiments were triplicated to ensure reproducibility.

In vivo analysis of carotenoid cleavage activity. For *in vivo* analysis, a synthetic module that expressed a C₃₀ or C₄₀ carotenoid (Table 2) and pUCM-NSC3 were cotransformed into *E. coli* JM109. As a negative control, an empty vector (pUCM) was transformed into *E. coli* JM109 expressing a synthetic carotenogenic module. More than 3 colonies of each *E. coli* transformant were cultivated overnight at 37°C in 4 ml LB medium supplemented with the appropriate antibiotics. The cultured cells were transferred to 100 ml Terrific broth medium and were further cultivated at 30°C for 24 h at 250 rpm. The cells were harvested by centrifugation (at

4°C and 4,000 rpm for 20 min), and their pigments were extracted repeatedly with a total volume of 10 ml acetone until the pigments were visibly removed. After centrifugation (at 4°C and 4,000 rpm), the colored supernatants were pooled and reextracted with an equal volume of hexane after the addition of an equal volume of deionized distilled water. The color fractions were then dried under nitrogen gas and were resuspended with 0.2 ml ethyl acetate. After centrifugation (at 13,000 rpm and 20 min), the extracts were analyzed by liquid chromatography-mass spectroscopy (LC-MS).

Production and purification of carotenoids. For the production of carotenoids, recombinant *E. coli* SURE or JM109 cells harboring a synthetic module expressing carotenoids were cultivated in 1 liter Terrific broth medium supplemented with the appropriate selective antibiotics (chloramphenicol at 50 μg/ml or kanamycin at 30 μg/ml) for 48 h at 30°C and 250 rpm. The cells were harvested via centrifugation (at 4°C and 8,000 rpm for 20 min), transferred to a glass tube, and suspended in 15 ml acetone. Extraction was carried out until the visible pigments were removed. In order to dehydrate the acetone extracts, they were all passed through sodium sulfate (anhydrous; Bio Basic). They were then subjected to silica gel chromatography and were eluted with solvent systems of hexane-ethyl acetate (9:1, vol/vol) or hexane-acetone (9:1, vol/vol) depending on the polarity of the carotenoid. The color fractions were then dried under nitrogen gas. Purified carotenoid samples were stored at -80°C until use.

HPLC and LC-MS analysis. Aliquots (10 to 20 μl) of the extracts from the *in vitro* or *in vivo* assays were applied to a Zorbax Eclipse XDB C₁₈ column (inner diameter, 4.6 mm; length, 150 mm; particle size, 5 μm; Agilent Technologies) and were eluted with the following solvent systems

TABLE 2 Plasmids used to supply carotenoids with various structures *in vivo*

Pathway	Plasmid	Genes expressed ^a	Major carotenoid(s)	Reference
C ₃₀	pAC-M _{SA} -N _{SA}	<i>crtM</i> , <i>crtN</i>	4,4'-Diaponeurosporene	19
	pAC-M _{SA} -N _{YSA} -Y _{tbl}	<i>crtM</i> , <i>crtN</i> , <i>crtYcYd_t</i>	4,4'-Diapotorulene, 4,4'-diaponeurosporene	Unpublished
	pAC-M _{SA} -N _{SA} -P _{SA}	<i>crtM</i> , <i>crtN</i> , <i>crtP</i>	4,4'-Diaponeurosporen-4'-al, 4,4'-diapolycopene-4,4'-dial	19
	pAC-M _{SA} -N _{SA} -P _{SA} + pUCM-Y _{tbl}	<i>crtM</i> , <i>crtN</i> , <i>crtP</i> , <i>crtYcYd_t</i>	4,4'-Diapotorulen-4'-al, 4,4'-diaponeurosporen-4'-al	Unpublished
	pAC-M _{SA} -N _{SA} -P _{SA} + pBBR-aldH _{SA}	<i>crtM</i> , <i>crtN</i> , <i>crtP</i> , <i>aldH</i>	4,4'-Diaponeurosporen-4'-oic acid, 4,4'-diaponeurosporen-4'-al	19
C ₄₀	pAC-E _{PA} -B _{PA} -I _{PA}	<i>crtE</i> , <i>crtB</i> , <i>crtI</i>	Lycopene	24
	pAC-E _{PA} -B _{PA} -I _{PA} -Y _{PA}	<i>crtE</i> , <i>crtB</i> , <i>crtI</i> , <i>crtY</i>	β-Carotene	24
	pAC-E _{PA} -B _{PA} -I _{PA} -Y _{PA} -Z _{PA}	<i>crtE</i> , <i>crtB</i> , <i>crtI</i> , <i>crtY</i> , <i>crtZ</i>	Zeaxanthin	24
	pAC-E-B-I ₁₄	<i>crtE</i> , <i>crtB</i> , <i>crtI₁₄</i>	3,4,3',4'-Tetrahydrolycopene	25
	pAC-E-B-I ₁₄ -Y ₂	<i>crtE</i> , <i>crtB</i> , <i>crtI₁₄</i> , <i>crtY₂</i>	Torulene	26

^a Here *crtP* encodes 4,4-diaponeurosporene oxidase in *Staphylococcus aureus*, not phytoene desaturase (*pds* or *crtP*) in cyanobacteria, algae, and plants.

by using an Agilent 1200 HPLC system equipped with a photodiode array detector: solvent A, acetonitrile-methanol (MeOH)-isopropyl alcohol (80:15:5, vol/vol/vol); solvent B, MeOH-water (70:30, vol/vol) with 0.1% ammonium acetate; solvent C, MeOH. To identify *in vivo* cleavage products, we used the gradient condition of 100% solvent B to 100% solvent C for 16 min, 100% solvent C for 19 min, 100% solvent C to 100% solvent A for 10 min, and 100% solvent A until 60 min or longer. The system was then returned to 100% solvent B at a flow rate of 0.8 ml/min. To identify the *in vitro* cleavage products, we used a gradient of 100% solvent B to 100% solvent C for 16 min, followed by 100% solvent C until 40 min, after which the system was returned to 100% solvent A at a flow rate of 0.8 ml/min. The gradients were extended for 2 to 3 min to resolve minor cleavage product peaks more clearly after complete conversion.

To analyze the kinetics results, we determined the reaction products by loading 30-μl extracts onto an HPLC system and typically eluting them under isocratic conditions with solvent system A at a flow rate of 1 ml/min. Mass fragmentation spectra were monitored using the positive ion modes in the mass range of *m/z* 100 to 800 on an Agilent 1200 LC-MS system equipped with an atmospheric pressure chemical ionization interface. For structural elucidation, the carotenoids and cleavage products were identified using a combination of HPLC retention times and UV/visible (UV/Vis) absorption and mass fragmentation spectra.

RESULTS

Cleavage of β-apo-8'-carotenal by NSC3. β-Apo-8'-carotenal was first used as a substrate for the *in vitro* characterization of NSC3. As reported by Marasco et al. (17), when 5 μg purified NSC3 is incubated with β-apo-8'-carotenal at 30°C, the reaction mixture changes from reddish to yellowish, whereas the reddish color of the control reaction mixture incubated without NSC3 remains unchanged (Fig. 2A, inset). LC-MS analysis of the reaction revealed 2 major peaks, at 20 min (Fig. 2A, peak b) and 21 min (Fig. 2A, peaks c and d), and 1 minor peak, at 19 min (Fig. 2A, peak e). Peak b was identified as C₂₀ *trans*-retinal based on the UV/Vis spectrum ($\lambda_{\max}$, 380 nm [Fig. 2B]), retention time (20 min), and molecular mass ($[M + H]^+$ at *m/z* = 285.2 [Fig. 2C]), all of which were the same as those of authentic *trans*-retinal. The formation of *trans*-retinal indicated that NSC3 cleaved the C-15=C-15' double bond in β-apo-8'-carotenal, a result that conflicts with the report of NSC3 cleavage activity at the C-9=C-10 double bond (17). Peaks c and d were identified as C₂₂ *cis*- and *trans*-β-apo-14'-carotenals based on their retention times, UV/Vis spectra (Fig. 2B), and molecular masses ($[M + H]^+$ at *m/z* = 311.4 [Fig. 2C]), indicating that NSC3 cleaved an additional, C-13'=C-14' double

bond in β-apo-8'-carotenal. Minor peak e was identified as C₁₈ β-apo-13-carotenone based on the UV/Vis spectrum and molecular mass ($[M + H]^+$ at *m/z* = 259.1 [Fig. 2C]), proving that NSC3 cleaved the C-13=C-14 double bond in β-apo-8'-carotenal as well. No reported apocarotenoid was detected as a cleavage product of the C-9=C-10 double bond in β-apo-8'-carotenal. Therefore, NSC3 can cleave the C-13=C-14, C-13'=C-14', and C-15=C-15' double bonds in β-apo-8'-carotenal *in vitro* (Fig. 1B), and multiple cleavage reactions can occur simultaneously or successively. *In vitro* assays with various incubation times have shown that NSC3 carries out simultaneous cleavage reactions rather than the successive cleavage of other CCDs (27).

Steady-state kinetic analysis of NSC3. Because no kinetic data on NSC3 comparable to those on NSC1 and NSC2 have been documented (17), enzymological characterization of NSC3 was performed using β-apo-8'-carotenal as a substrate. Optimal reaction conditions for NSC3 were 30°C and pH 7.0 based on the relative activity of NSC3 and the thermal stability of β-apo-8'-carotenal (approximately 5% of β-apo-8'-carotenal decomposes to unknown products in 30 min in the absence of NSC3). The *K_m* and *V_{max}* values of NSC3 on β-apo-8'-carotenal were 27.8 ± 3.4 μM and 0.29 ± 0.03 μM/min, respectively. The *K_m* value of NSC3 on β-apo-8'-carotenal was similar to that of NSC2 (16), whereas *k_{cat}* and *k_{cat}/K_m* values were 51.54 ± 5.41 s⁻¹ and 1.85 ± 0.04 μM⁻¹ s⁻¹, respectively, and reflected fast catalytic activity on β-apo-8'-carotenal compared to that of other CCDs or apocarotenal cleavage oxygenases (12).

Cleavage of C₃₀ carotenoids by NSC3 *in vivo*. To investigate the cleavage reaction of NSC3 *in vivo*, we examined 6 C₃₀ carotenoids with natural and nonnatural structures as the substrates in recombinant *E. coli* strains metabolically engineered to produce C₃₀ carotenoids with various structures. Basically, to supply C₃₀ carotenoids *in vivo*, plasmids with synthetic modules expressing C₃₀ carotenoids with various structures (16, 23) were cotransformed into *E. coli* expressing NSC3. Acyclic C₃₀ carotenoids (4,4'-diaponeurosporene, 4,4'-diapolycopene-4,4'-dial, 4,4'-diaponeurosporen-4'-al, and 4,4'-diaponeurosporen-4'-oic acid) (Table 2) were examined first *in vivo*. NSC3 cleaved 4,4'-diaponeurosporene at the C-13'=C-14' double bond, and the resulting cleavage product was identified as C₁₇ apo-14'-diaponeurosporene (Fig. 3A, peak b). NSC3 also cleaved the monoaldehyde caro-

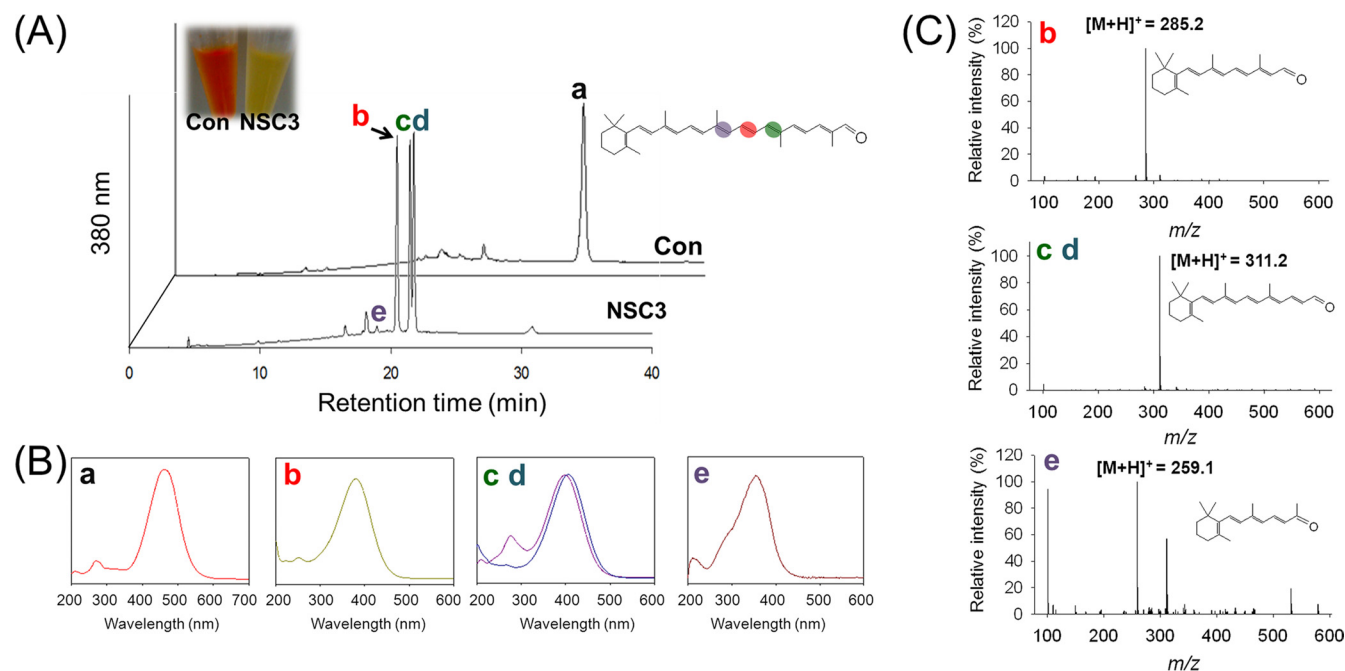


FIG 2 Cleavage of β -apo-8'-carotenal by NSC3. (A) High-performance liquid chromatography analysis of crude extracts obtained from NSC3 assay mixtures. The control reaction mixture (Con) was incubated without NSC3. (Inset) Changed color of the reaction mixture. (B and C) UV/Vis (B) and MS (C) spectra of identified peaks were recorded. Individual peaks were identified as follows: peak a, β -apo-8'-carotenal; peak b, *trans*-retinal; peaks c and d, *cis*- and *trans*- β -apo-14'-carotenal; peak e, β -apo-13-carotenone.

tenoid 4,4'-diaponeurosporen-4'-al at the C-9'=C-10' double bond and produced C_{22} apo-10'-diaponeurosporenal as a cleavage product (Fig. 3B, peak e). However, NSC3 did not cleave the dialdehyde carotenoid 4,4'-diapolycopene-4,4'-dial. The carboxylic carotenoid 4,4'-diaponeurosporen-4'-oic acid seemed to be cleaved at the C-9'=C-10' double bond by NSC3, because C_{22} apo-10'-diaponeurosporenal was detected as a cleavage product (Fig. 3C, peak e). Although C_{22} apo-10'-diaponeurosporenal may be a cleavage product of 4,4'-diaponeurosporen-4'-al (Fig. 3C, peak c), because the synthetic module that expressed 4,4'-diaponeurosporen-4'-oic acid also produced 4,4'-diaponeurosporen-4'-al as a minor product in *E. coli* (Table 2), the subsequent *in vitro* study confirmed that 4,4'-diaponeurosporen-4'-oic acid was clearly cleaved by NSC3 (see Fig. 5C).

Next, 2 nonnatural monocyclic C_{30} carotenoids (4,4'-diapotorulene and 4,4'-diapotorulen-4'-al) were examined *in vivo*. These carotenoids have not been found in nature and were synthesized in *E. coli* using 2 enzymes generated via directed evolution: a mutant diapophytoene desaturase and a mutant lycopene cyclase (unpublished data). NSC3 cleaved the monocyclic carotenoid 4,4'-diapotorulene at its C-13'=C-14' double bond and the monocyclic aldehyde carotenoid 4,4'-diapotorulen-4'-al at its C-9'=C-10' double bond. The cleavage products were identified as C_{17} apo-14'-diapotorulenal (Fig. 3D, peak h) and C_{22} apo-10'-diapotorulenal (Fig. 3E, peak j) based on their retention times, UV/Vis spectra, and molecular masses ($[M + H]^+ = 245.2$ and 311.2, respectively).

Cleavage of C_{40} carotenoids by NSC3 *in vivo*. For further investigation of the cleavage reaction of NSC3 *in vivo*, 5 C_{40} carotenoids with different structures were examined as substrates in *E. coli*. In a manner similar to that for supplying C_{30} carotenoids *in*

vivo, plasmids with synthetic modules expressing the C_{40} carotenoids were cotransformed into *E. coli* expressing NSC3. First, 2 acyclic C_{40} carotenoids (lycopene and 3,4,3',4'-tetrahydrolycopene [Table 2]) were examined *in vivo*. While NSC3 did not cleave lycopene (Fig. 4A), it cleaved a fully conjugated carotenoid, 3,4,3',4'-tetrahydrolycopene, to generate cleavage products (Fig. 4B, peaks b and c). Although highly resolved MS spectra of the cleavage products could not be obtained because of the small quantities present, they were highly likely to be the cleavage products of 3,4,3',4'-tetrahydrolycopene, since their UV/Vis spectra were similar to those of apocarotenals. Next, 3 cyclic C_{40} carotenoids (torulene, β -carotene, and zeaxanthin) were examined *in vivo*. NSC3 cleaved the monocyclic and fully conjugated carotenoid torulene (Fig. 4C, peaks e and f), and one of the cleavage products was determined to be C_{22} *trans*-retinal, which indicated that NSC3 cleaved the C-15=C-15' bond in torulene. However, NSC3 did not cleave the bicyclic carotenoids β -carotene and zeaxanthin (Fig. 4D and E).

Cleavage of C_{30} and C_{40} carotenoids by NSC3 *in vitro*. After several C_{30} and C_{40} carotenoids were examined as *in vivo* substrates of NSC3, the *in vitro* cleavage reaction of NSC3 was investigated using 5 purified C_{30} carotenoids (4,4'-diaponeurosporene, 4,4'-diaponeurosporen-4'-al, 4,4'-diaponeurosporen-4'-oic acid, 4,4'-diapotorulene, and 4,4'-diapotorulen-4'-al) and 1 C_{40} carotenoid (torulene). As observed *in vivo*, the main cleavage product of 4,4'-diaponeurosporene was C_{17} apo-14'-diaponeurosporenal (Fig. 5A, peak a-1). Notably, an additional cleavage product (C_{13} apo-13'-diaponeurosporenone), which was not detected *in vivo*, was also observed (Fig. 5A, peak a-2). These results support the hypothesis that NSC3 cleaves 4,4'-diaponeurosporene at the C-13'=C-14' double bond. Similarly, the cleavage products

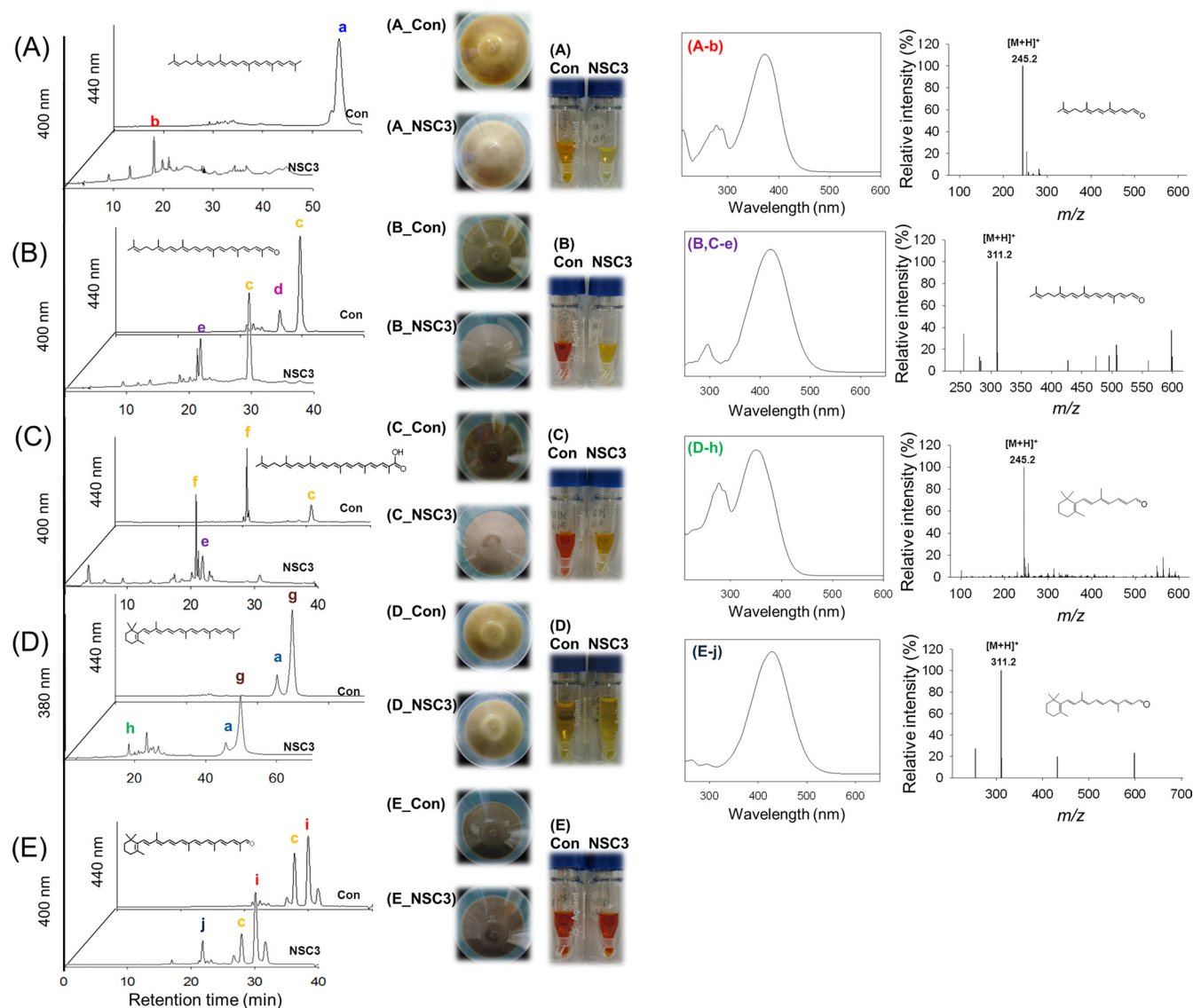


FIG 3 Cleavage of C_{30} carotenoids with different structures by NSC3 *in vivo*. From left to right, high-performance liquid chromatograms, cell pellets, crude extracts, UV/Vis spectra, and MS spectra of cleavage reaction products from *Escherichia coli* expressing synthetic modules and NSC3 together are shown. (A) pAC- M_{SA} - N_{SA} ; (B) pAC- M_{SA} - N_{SA} - P_{SA} ; (C) pAC- M_{SA} - N_{SA} - P_{SA} + pBBR-aldH $_{SA}$; (D) pAC- M_{SA} - N_{YSA} - Y_{tBL} ; (E) pAC- M_{SA} - N_{SA} - P_{SA} + pUCM- Y_{tBL} . The individual peaks were identified as follows: peak a, 4,4'-diaponeurosporene; peak b, apo-14'-diaponeurosporene; peak c, 4,4'-diaponeurosporen-4'-al; peak d, 4,4'-diapolycopene-4,4'-dial; peak e, apo-10'-diaponeurosporene; peak f, 4,4'-diaponeurosporen-4'-oic acid; peak g, 4,4'-diapotorulene; peak h, apo-14'-diapotorulene; peak i, 4,4'-diapotorulen-4'-al; peak j, apo-10'-diapotorulene.

of 4,4'-diaponeurosporen-4'-al were identified as C_{22} apo-10'-diaponeurosporene (Fig. 5B, peak b-1), which was also observed *in vivo*, and C_{17} apo-14'-diaponeurosporene (Fig. 5B, peak b-2), which was absent *in vivo*. In contrast to the single cleavage of 4,4'-diaponeurosporene at the C-13'=C-14' double bond, 4,4'-diaponeurosporen-4'-al was cleaved at both the C-9'=C-10' and C-13'=C-14' double bonds. However, as observed *in vivo*, only C_{22} apo-10'-diaponeurosporene (Fig. 5C, peak c-1) was detected as a cleavage product of 4,4'-diaponeurosporen-4'-oic acid at the C-9'=C-10' double bond. Similarly, the monocyclic carotenoid 4,4'-diapotorulene was cleaved *in vitro* at the C-13'=C-14' double bond, just as it was *in vivo*, resulting in C_{17} apo-14'-diapotorulene (Fig. 5D, peak d-1). The monocyclic carotenoid 4,4'-diapotorulen-4'-al was also cleaved at the C-9'=C-10' double bond

in vitro, and C_{22} apo-10'-diapotorulene, which was observed *in vivo*, was identified as a cleavage product (Fig. 5E, peak e-1). The longer monocyclic carotenoid torulene was also cleaved at the C-15=C-15' double bond, generating C_{20} *trans*-retinal (Fig. 5F, peak f-1), as observed *in vivo*.

DISCUSSION

CCDs from various sources have been cloned and identified functionally to elucidate their physiological roles in plants and animals (27). *Nostoc* sp. PCC 7120 is a well-known cyanobacterium in which 3 CCDs have been identified thus far: NSC1, NSC2, and NSC3. Enzymatic knowledge of NSC1 and NSC2 is thorough, but the enzymatic properties of NSC3 remain unclear.

We observed that NSC3 cleaves β -apo-8'-carotenal at 3 spe-

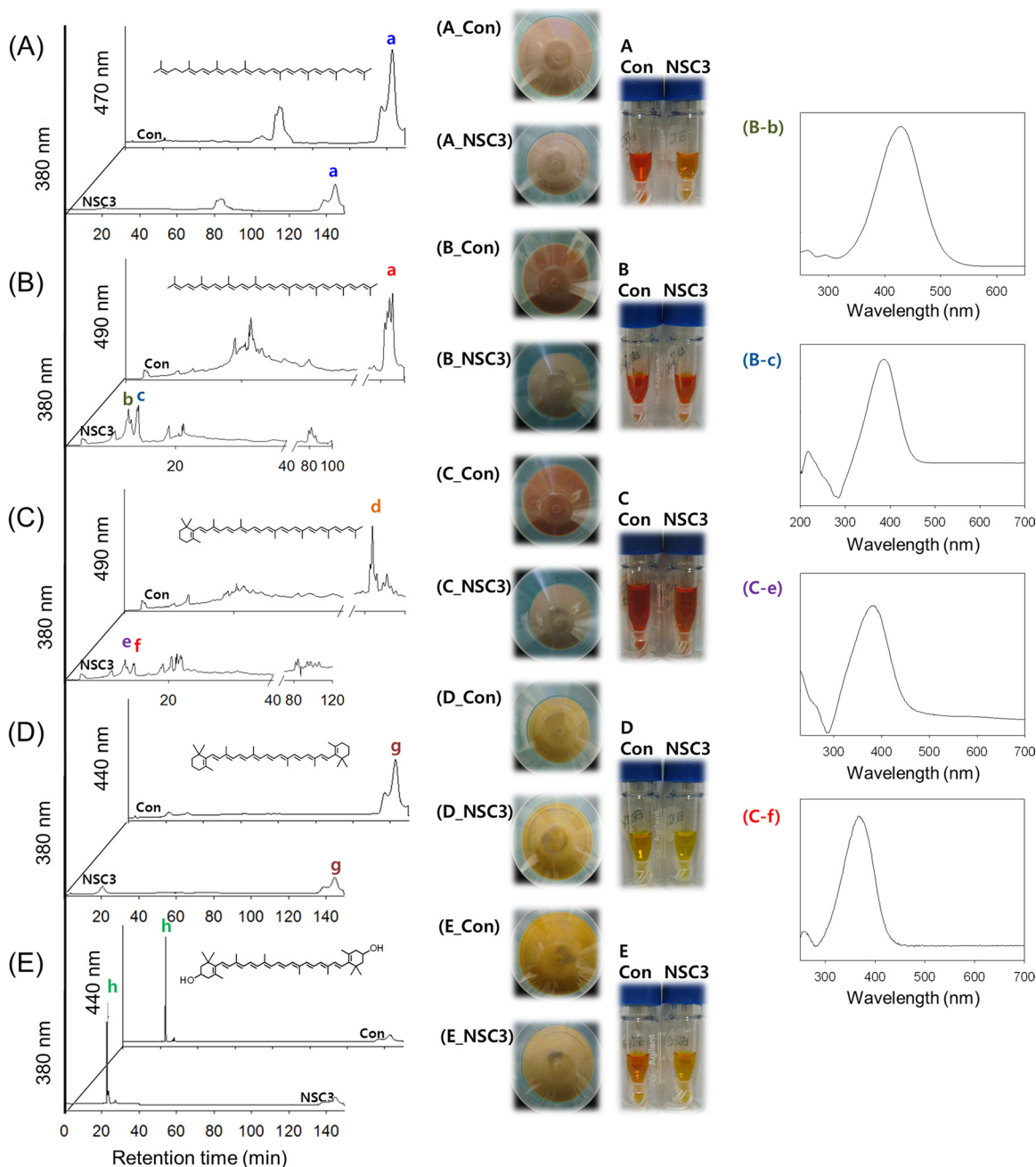


FIG 4 Cleavage of C_{40} carotenoids with different structures by NSC3 *in vivo*. From left to right, high-performance liquid chromatograms, cell pellets, crude extracts, and UV/Vis spectra of cleavage reaction products from *E. coli* coexpressing synthetic gene modules and NSC3 are shown. (A) pAC-E_{PA}-B_{PA}-I_{PA}; (B) pAC-E-B-I₄; (C) pAC-E-B-I₄-Y₂; (D) pAC-E_{PA}-B_{PA}-I_{PA}-Y_{PA}; (E) pAC-E_{PA}-B_{PA}-I_{PA}-Y_{PA}-Z_{PA}. The peaks were identified as follows: peak a, lycopene; peaks b, c, e, and f, unidentified cleavage products; peak d, torulene; peak g, β -carotene; peak h, zeaxanthin.

cific double bond positions *in vitro*—C-15=C-15', C-13'=C-14', and C-13=C-14—and has relatively broad preferences for natural or nonnatural carotenoid structures *in vivo* and *in vitro*. NSC1 catalyzes the cleavage of C-9=C-10 and C-9'=C-10' bonds in bicyclic carotenoids and the cleavage of C-9=C-10 and C-7'=C-8' bonds in monocyclic carotenoids (15, 17). NSC2 cleaves β -apo-8'-carotenol or β -carotene at their C-15=C-15' bonds, as well as carotene backbones with chain lengths of C_{27} to C_{30} , carotene backbones of hydroxylated β -rings, and carotene

backbones of substituted aldehyde-to-hydroxyl groups at the C-15=C-15' double bond (15). Therefore, NSC1, NSC2, and NSC3 in *Nostoc* sp. PCC 7120 may function cooperatively in response to cellular conditions, because they have relatively different substrate preferences and cleavage reactions.

The observed cleavage pattern of NSC3 is unique because the cleavage of C-13'=C-14' double bonds in carotenoids has been observed mainly in animal intestinal enzyme extracts with β -apo-8'-carotenol or β -carotene as the substrate (28, 29). Although the

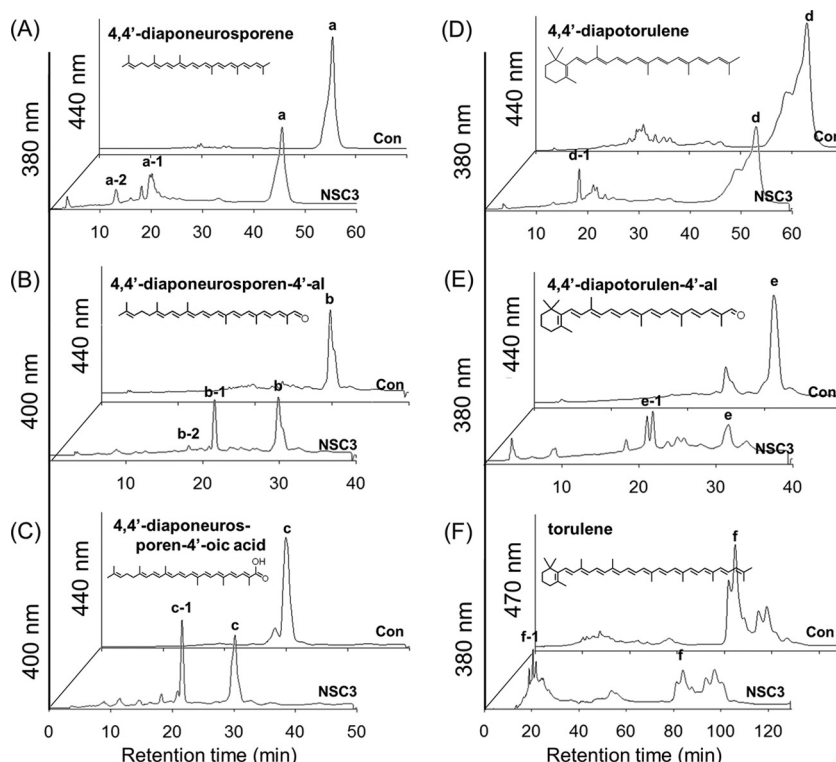


FIG 5 Cleavage of purified C_{30} and C_{40} carotenoids with different structures by NSC3 *in vitro*. High-performance liquid chromatograms of reaction products from an *in vitro* assay of NSC3 cleavage of purified carotenoid structures are shown. (A) 4,4'-Diaponeurosporene (peak a); (B) 4,4'-diaponeurosporen-4'-al (peak b); (C) 4,4'-diaponeurosporen-4'-oic acid (peak c); (D) 4,4'-diapotorulene (peak d); (E) 4,4'-diapotorulene-4'-al (peak e); (F) torulene (peak f). The cleavage peaks were identified as follows: a-1, apo-14'-diaponeurosporenal ($\lambda_{\max}$ 373 nm; M^+ at m/z = 244.18); a-2, apo-13'-diaponeurosporenone ($\lambda_{\max}$ 370 nm; M^+ at m/z = 190.13); b-1, apo-10'-diaponeurosporenal ($\lambda_{\max}$ 422 nm; M^+ at m/z = 310.23); b-2, apo-14'-diaponeurosporenal; c-1, apo-10'-diaponeurosporenal; d-1, apo-14'-diapotorulenal ($\lambda_{\max}$ 355 nm; M^+ at m/z = 244.18); e-1, apo-10'-diapotorulenal ($\lambda_{\max}$ 408 nm; M^+ at m/z = 310.23); f-1, retinal ($\lambda_{\max}$ 380 nm; M^+ at m/z = 284.21).

cellular metabolism of the C-13'=C-14' cleavage product β -apo-14'-carotenal has not been documented in *Nostoc* sp. PCC 7120, NSC1 or NSC2 may be involved in the oxidative cleavage reaction of β -apo-14'-carotenal to generate retinal or other signaling compounds, such as abscisic acid or trisporoids in *Blakeslea trispora* (30). In addition, the cleavage of C-13=C-14 double bonds has been reported only for the CCDs of *Arabidopsis thaliana*, *Oryza sativa*, *Pisum sativum*, *Novosphingobium aromaticivorans*, and *M. tuberculosis* (9, 11, 13, 31). In particular, the carotenoid cleavage oxygenase of *M. tuberculosis* (MtCCO) cleaves C-13=C-14 and C-13'=C-14' double bonds as well as C-15=C-15' double bonds in various bicyclic carotenoids, including β -carotene or apocarotenals (13). However, although NSC3, like MtCCO, cleaves the C-13=C-14, C-13'=C-14', and C-15=C-15' double bonds in β -apo-8'-carotenal, it cannot cleave bicyclic carotenoids, such as β -carotene. Structural studies will elucidate the differences in substrate preference between MtCCO and NSC3.

NSC3 has a relatively broad preference for carotenoid structures. It can cleave both acyclic C_{30} carotenoids (4,4'-diaponeurosporene, 4,4'-diaponeurosporen-4'-al, and 4,4'-diaponeurosporen-4'-oic acid) and monocyclic C_{30} carotenoids (4,4'-diapotorulene and 4,4'-diapotorulene-4'-al) *in vivo* (Fig. 6). The *in vitro* assay using purified C_{30} carotenoids demonstrated the same unique NSC3 activities observed *in vivo*. NSC3 also cleaves both the acyclic fully conjugated C_{40} carotenoid 3,4,3',4'-tetrahydrolycopene and the monocyclic fully conjugated C_{40} carotenoid torulene *in vivo*

and *in vitro* (Fig. 6). In the latter case, the C-15=C-15' cleavage product retinal was generated from torulene by a direct cleavage reaction of NSC3 at the C-15=C-15' double bond. However, NSC3 cannot accept other C_{40} carotenoids, such as lycopene and β -carotene, which have no fully conjugated double bonds in at least one end of their backbones (Fig. 6). This NSC3 substrate preference may be explained by the fact that the fully conjugated double bonds of a carotene backbone would aid in the entry and binding of the carotenoid into an active pocket (32, 33).

The unique cleavage activity of NSC3 can be used to produce apocarotenals of biotechnological importance. Recently, apocarotenals and apocarotendials have attracted increasing interest owing to their anticancer effects (34, 35). Therefore, the cleavage products of C_{30} carotenoids, such as C_{17} β -apo-14'-diaponeurosporenal, reported in this study may be bioactive compound candidates in, for example, electrophile/antioxidant response element transcription systems (34). In addition to these biological activities, β -apo-14'-carotenal has been discovered to inhibit the activation of peroxisome proliferator-activated receptors and retinoid X receptor in adipocytes (35).

We conclusively characterized the activity and substrate preferences of NSC3 *in vitro* and *in vivo* with natural and nonnatural carotenoid structures generated with synthetic carotenoid modules in *E. coli*. NSC3 displays unique cleavage activity with β -apo-8'-carotenal *in vitro*, providing new insight into the cleavage reactions of CCDs. We also applied synthetic biology to an inves-

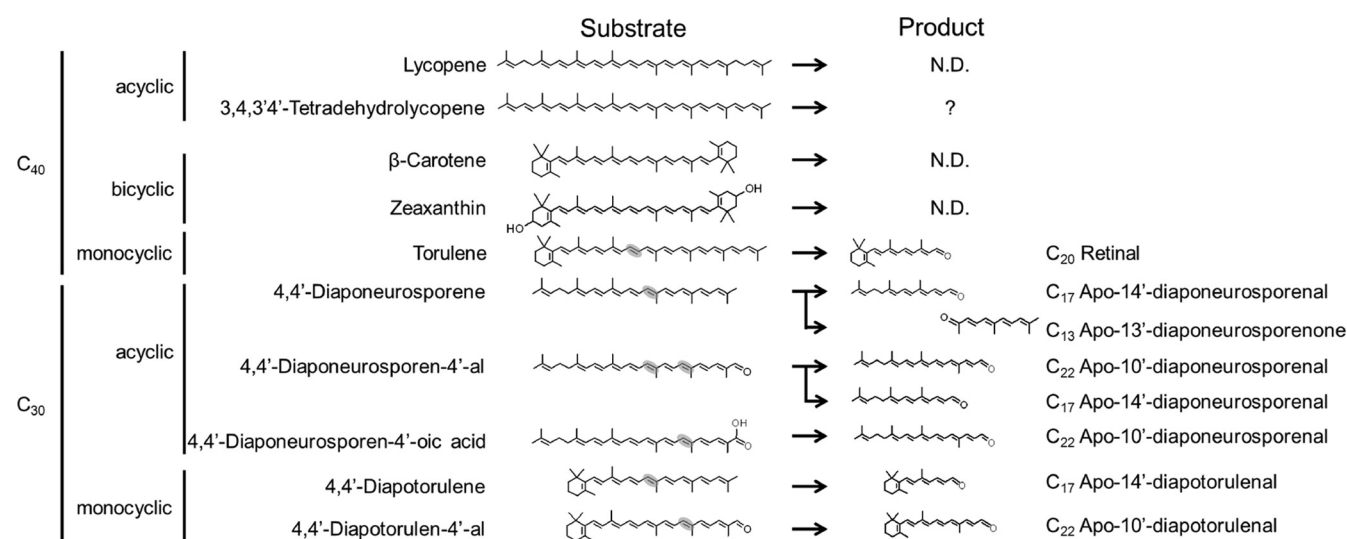


FIG 6 Structures of C₃₀ and C₄₀ carotenoids that serve as substrates for NSC3 and their cleavage products. The cleavage sites in each carotenoid substrate are indicated by shaded ellipses. ND, not detected; ?, high possibility but no mass data.

tigation of the cleavage reactions and substrate preferences of NSC3 *in vivo* and *in vitro*. The modular expression of carotenoid pathway enzymes in *E. coli* provides NSC3 with natural and non-natural carotenoid structures *in vivo* and *in vitro*. Consequently, we found that NSC3 uses natural and nonnatural C₃₀ carotenoids (not apocarotenoids) as substrates to generate structurally novel cleavage products. These novel apocarotenal structures may be bioactive compounds of biotechnological significance. In addition to the potential biotechnological applications of their novel cleavage products, carotenoids with natural or nonnatural structures can also provide information valuable for understanding the cleavage reactions and substrate preferences of other CCDs *in vivo* and *in vitro*.

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