6. Plant carotenoid cleavage oxygenases activities

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Abstract

Carotenoid cleavage products, apocarotenoids, are biologically active compounds exerting important functions as chromophor, hormones, signaling molecules, volatiles, and pigments. Apocarotenoids are generally synthesized by the carotenoid cleavage dioxygenases (CCDs) that comprise a ubiquitous family of enzymes. The activity of plant CCDs was unraveled more than 20 years ago, with the characterization of the maize VP14, the first identified CCD. The protocol developed to determine the activity of this enzyme *in vitro* is still being used, with minor modifications. In addition, *in vivo* procedures have been developed during these years, mainly based on the exploitation of *Escherichia coli* cells engineered to produce specific carotenoid substrates. Further, technological developments have led to significant improvements, contributing to a more efficient detection of the reaction products**.** This article provides an updated set of detailed protocols suitable for the *in vitro* and *in vivo* characterization of the activities of CCDs, starting from well-established methods.

Key words: apocarotenoids, carotenoids, CCD, enzymatic activity,

1. Introduction

In all clades of life, enzymatic breakage of carotenoids gives rise to biologically active molecules, known as apocarotenoids. In plants, apocarotenoids play important functions, which include their role as hormones, such as abscisic acid (ABA) and strigolactones (SL) [1-3] and as signals regulating different developmental processes [4-6] and involved in thermo-tolerance and adaptation to environmental stress [17,18]. In addition, apocarotenoids contribute plant-to-plant communication [15,16], and to herbivore and pathogen defense [7] [8] [9,10], and attract animals for pollination and seed dispersal [11,12] [13,14].

Carotenoid cleavage, the initial step in the biosynthesis of apocarotenoids, is generally mediated by non-heme iron (II) enzymes, the carotenoid cleavage dioxygenases (CCDs) that catalyze the oxidative cleavage of carbon-carbon double bonds in carotenoid backbones, generating products with aldehyde or ketone end groups [19]. Plants CCDs enzymes are classified into two big subfamilies [20]. The nine-*cis*-epoxy-carotenoid-dioxygenases (NCED) subfamily represented by the first characterized CCD, the maize VP14 [1], that catalyzes the synthesis of the ABA precursor xanthoxin by cleaving the C11,C12 double bond in 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin. NCEDs are represented in practically all plant species by a multigene family [21]. The second subfamily is generally referred to as CCD, regardless the type of carotenoid substrates and the cleavage site, and includes all other members of the CCD family, which are not involved in ABA biosynthesis. Indeed, plant CCDs cleave a variety of carotenoid and apocarotenoid substrates at different positions [22-28]. The plant CCD subfamily includes CCD1, CCD2, CCD4, CCD7 and CCD8 enzymes [20]. Large scale genomic sequencing projects on plant species has allowed the identification of many putative

CCDs that have expanded this subfamily. However, biochemical studies have only been reported for a limited number of CCDs [20,29].

Two main strategies, *in vitro* and/or *in vivo* studies, have been followed to characterize the activity of CCD enzymes. These studies have been performed with CCDs from many plant species, including Arabidopsis [30], saffron [31], rose [32], petunia [33], tomato [34,35], potato [22], rice [36], melon [37], maize [38], Vitis [39,40], citrus [26,41] and other plant species [42]. The *in vivo* studies have been done by expressing CCDs in carotenoid accumulating *E. coli* strains containing specific plasmids that allowed the accumulation of different carotenoids substrates (Table 1). The *in vitro* approach allows testing of much more substrates, including apocarotenoids themselves, and enables comprehensive and quantitative characterization of investigated enzymes. In both cases, products formed have been detected and identified through thin-layer chromatography (TLC), HPLC, LC-MS or GC-MS.

2. Materials

2.1 *In vivo* assays

1. Bacterial strains for carotenoid production. Most common *E. coli* strains, such as JM109, XL1-Blue. TOPO-10, used for cloning are utilized to accumulate carotenoids.

2. Plasmids responsible for carotenoid production are listed in (Table 1). These plasmids can be purchased from addgene (https://www.addgene.org).

3. Growth media for *E. coli* cells. Luria bertani (LB) liquid (combine 10 g triptone, 10 g NaCl and 5 g yeast extract in 900 mL ddH_2O and shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the final volume of the solution to 1 L with ddH_2O. Sterilize by autoclaving for 20 min at 15 psi) and solid LB media, has the same composition of liquid LB plus 15 g of agar.

4. Antibiotics stocks. Ampicillin, carbenicillin and kanamycin are dissolved in ddH_2O at a concentration of 100 mg/mL for ampicillin and 50 mg/mL for carbenicillin and kanamycin. Chloramphenicol is dissolved in EtOH at a concentration of 50 mg/mL.

5. Organic solvents for carotenoids and apocarotenoids extraction and analyses. Acetone, MeOH, $CHCl_3$, $CHCl_2$.

6. Specific oligonucleotides for cloning in an expression vector the CCD to be characterized.

7. Expression vectors. Most frequently used expression vectors are shown in Table 2.

8. 20% Arabinose and 1mM isopropyl β-d-thiogalactopyranoside (IPTG). Both dissolved in ddH_2O and sterilize by filtration.

9. Nitrogen.

10. For HPLC and GC analyses you will need appropriated vials and columns for carotenoid analyses and solvents.

2.2 *In vitro* assays

1. Bacterial strains. Most common used *E. coli* strains BL21 DE3pLysS, BL21A1, and XL1-Blue.

2. Plasmid pGro7 encoding the groES-groEL-chaperone system is used to improve protein folding.

3. Growth media for *E. coli* cells and antibiotics is in item 3 in section 2.1 *In vivo* assays. In addition, the 2x YT media (dissolve 16 g tryptone, 5 g NaCl and 10 g yeast extract in 900 mL ddH_2O, adjust the pH to 7.0 with 5 N NaOH and the final volume of the solution to 1 L with ddH_2O. Sterilize by autoclaving for 20 min at 15 psi).

4. Purified carotenoid and apocarotenoid substrates.

5. Prepare the carotenoid and or apocarotenoid substrates for the assays (see Note 4). Resuspend the substrates in 200 µL of benzene (or dicloromethane) at a final concentration of 50 to 100 µM and mixed with 150µl of an ethanolic detergent mixture consisting of 0.7% (v/v) Triton X-100 and 1.6% (v/v) Triton X-405, for lycopene and pro-lycopene substrates or with 250 µl of an ethanolic β-octylglucoside solution (4% w/v) for apocarotenoids and other carotenoids. The final mix is evaporated to dryness in a vacuum centrifuge to get a carotenoid gel on the bottom of the tube. (see Note 5).

6. Modified 2x Lysis-Equilibration-Wash buffer (LEW) (100 mM NaH_2PO_4, 600 mM NaCl, pH 8.0 using NaOH), containing 1 mg/mL lysozyme, 0.1% Triton X-100 and 1 mM DTT.

7. Triton X-100, Triton X-405 and octyl-β-glucoside.

8. Organic solvents for extraction and substrates preparation: acetone, benzene, $CHCl_2$, $CHCl_3$, petroleum, ether petroleum, and diethyl ether.

9. Expression vectors. Most frequently used expression vectors are present in Table 3.

10. 20% Arabinose and 1mM isopropyl β-d-thiogalactopyranoside (IPTG). Both dissolved in ddH_2O and sterilize by filtration.

10. Assay incubation buffer, 2X: 2 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 0.4 mM $FeSO_4$, 200 mM HEPES-NaOH pH 7.8, and 2 mg mL/L catalase.

11. For HPLC and GC analyses you will need appropriated vials and columns for carotenoid analyses and solvents.

2.3. Equipment

Table centrifuge

Shaker with controlled temperature

Spectrophotometer

French pressure cell or an ultrasonic homogenizer

Vacuum centrifuge

Nitrogen

Balance

HPLC-PDA

HPLC-PDA-MS

GC-MS

3. Methods

3.1 *In vivo* assays

1. Create a stock of *E. coli* cells producing different carotenoid substrates to use for your assays. Transform competent cells of the selected *E. coli* strain with each of the pAC vectors, by using standard transformation procedure, such as electroporation [344] or heat-shock [44]. Grow individual colonies to prepare glycerol stocks

2. Make competent *E. coli* strains engineered to accumulate different carotenoid substrates by a standard procedure (http://cshprotocols.cshlp.org/) and keep them at -80 °C.

3. Transform the competent *E. coli* strains engineered to accumulate different carotenoid substrates with the selected expression vectors harboring the CCD under study (see Notes 1 and 2) and the empty vector, which is used as a negative control of activity.

4. Take individual colonies to initiate a pre-culture in a volume of 3 mL LB containing the appropriate antibiotics, and grow overnight at 37 °C.

5. Use 2.5 mL of the overnight cultures to inoculate 50 ml of LB with final concentration of antibiotics reduced to the half, in 200 ml flasks. Grow bacteria at 37 °C

with shaking (180 rpm) to an OD 600 nm of 0.5, and induced with the appropriate agent (arabinose or IPTG), depending on the expression vector selected (Table 2). Incubate the cultures at 28, 20 °C or 16 °C overnight in the dark (see Note 3).

6. Transfer the content of the flask to 50 mL tubes and harvest the *E. coli* cells by centrifugation. The color intensities of the resulting bacterial pellets will be compared by visible inspection to control bacterial cells harboring the empty expression plasmid. Changes in intensities is in many cases an indicator for CCD activity. However, it has been also observed that the expression of the CCD interferes with carotenoid biosynthesis, causing color change without cleavage activity. (you always need a product to be sure that you have a cleavage enzyme and the right substrate).

7. For pigment extraction for HPLC analyses, add 2.5 mL acetone to the bacterial pellet and vortex until total resuspension. Centrifuge al 9,000g for 10 min and collect the liquid part. If the pellet remain pigmented repeat again the MeOH:$CHCl_3$ extraction. Combine all the extracts and dry under nitrogen. In addition after the first acetone extraction, the obtained pellet could be extracted by adding to the pellet 2.5 mL of MeOH:$CHCl_3$ (1:1), and vortex until total resuspension. Centrifuge al 9,000g for 10 min and collect the liquid.

8. Resuspend the dry residue in $CHCl_3$, $CHCl_2$ or acetone and stored at -80 °C prior to HPLC-PDA or HPLC-PDA-MS analyses.

9. For volatile analyses, after induction of the cultures (point 5) transfer 5 mL to a tightly closed 50 mL tube. Incubate the tubes at 16 °C for 20 h (125 rpm). An SPME fiber (65 µm polydimethylsiloxane-divinylbenzene) is introduced into the vial through a septum and the headspace volatiles are allowed to be absorbed by the fiber at 45 °C for 30 min. The SPME fiber is keep at -80 °C until the GC analyses.

3.2 *In vitro* assays

1. Amplified the CCD sequence with the corresponding specific oligonucleotides by PCR and cloned it in the selected expression vectors.

2. Transform the competent *E. coli* strain selected with the expression vectors harboring the CCD under study (see Notes 1, 2 and 6) and the empty vector that is used as a negative control of activity. (see Note 5)

3. Take individual colonies to initiate a pre-culture in a volume of 3 mL LB containing the appropriate antibiotics, and grow overnight at 37 °C.

4. Use 2.5 mL of the overnight cultures to inoculate 50 ml of 2xYT with the appropriate antibiotics at half strength. Grow bacteria at 28 °C with shaking (150 rpm) to an OD 600 nm of 0.7, and induced with the appropriate agent (arabinose or IPTG), depending on the expression vector selected (Table 3). Incubate the cultures at 28 °C with shaking (150 rpm) for 4-6h or overnight at 18-20°C (see Note 3).

5. Harvest the *E. coli* cells by centrifugation for 6 min at 6000g. Resuspend the cell pellet in 1 mL 2x LEW buffer, and incubate the tubes for 30 min on ice. Disrupt the cells by sonication, French pressing or other preferred method [45], and centrifuged at 12000g for 30 min at 4 °C. Use the isolated supernatant for the *in vitro* assays.

6. For the *in vitro* assay. In a total volume of 200 µL, the carotenoid gel is resuspended by vortex in 100 µL 2x assay incubation buffer (single assay) and sonicated to obtain a clear micellar solution. Add 50 µL of crude protein lysate and ddH_2O to adjust at 200 µL. Transfer the tubes into a shaker (200 rpm) at 28-30 °C in darkness for 2-6 h or overnight. The reaction can be follow by visible inspection of the crude protein containing your CCD to the control extracts. Changes in color indicate activity of the CCD.

7. For HPLC analysis, the assay is stopped by adding 400 µL of acetone, vortex, and add 600 µL petroleum ether : diethyl ether (1:4), and vortex again. Centrifuge the tube at 12.000g x 1 min. After centrifugation, there are two phases visible. Take the epiphase, and transfer it to a new clean tube. Repeat this step with the lower aqueous phase. Combine the two the epiphase s and dry in a vacuum centrifuge. Samples can be store at -80 °C until until HPLC-PDA or HPLC-PDA-MS analyses.

8. For GC analyses, the assay is perform in screw-top GC glass vials, and volatiles are collected for 30 min after the reaction incubation time, using SPME (solid phase microextraction) fibers (100 µm polydimethylsiloxane).

4. Notes

1. Removal of signal peptides may improve the enzymatic activity, as it can interfere with the fold of the CCD and with its activity.

2. At the time of selection of the expression vector, some researches prefer to choose a vector with a fusion partners or tags to improve protein production yields, solubility and folding, which result in higher activity. CCDs are problematic to express in bacteria cells as they tend to be insoluble.

3. Incubation can be done the first time at 28 °C for 4 h. Although longer incubation times at lower temperature improve the final result. Dark conditions prevent possible photo-degradation of carotenoids.

4. Carotenoid and apocarotenoids can be purchase from commercial providers, but also you can purified by your own the substrates you need. For most of the carotenoid substrates, you can use the bacterial strains that accumulate the different carotenoids and purified them by TLC. For apocarotenoids or most specific carotenoids substrates don't produced by the bacterial strains, you can use the plants known to accumulate those metabolites and proceed with the isolation and purification as referred in the corresponding literature associated to those compounds.

5. Carotenoid and apocarotenoid substrates differ in their solubility in organic substrates, and different detergent. In addition, the detergent can have an effect on the enzyme activity. Different studies have used solvents such as acetone, benzene, hexane, and chloroform and detergents such as Triton X-100, Tween 20, Tween 80, Span 20, Span 80, and β-octylglucoside to form micelles of carotenoids under aqueous conditions. Thus, the formation of detergent micelles of a specific carotenoid or apocarotenoid substrate under aqueous conditions to be used for in vitro conditions will need optimization.

6. Several strategies have been made for efficient production of proteins in *E. coli*, like the co-production of chaperones to help correct protein fold. The co-transformation of E.coli cells with the plasmid pGro7, encoding the groES-groEL-chaperone system, could improve protein folding and further enzyme activity.

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Table 1. Plasmids for carotenoids production in *E.coli*.

Plasmid name	Genes present	Carotenoid produced
pAC-PHYTipi	contains crtE, crtB, and idi genes of *Erwinia herbicola* (*Pantoea agglomerans*) Eho10	phytoene
pAC-ZETAipi	contains crtE, crtB, and idi genes of *Erwinia herbicola* (*Pantoea agglomerans*) Eho10, and crtP gene of *Synechococcus* PCC7942	ζ-carotene
pAC-NEUR	contains the *Erwinia herbicola* (*Pantoea agglomerans*) Eho10 genes crtE and crtB, and the *Rhodobacter capsulatus* crtI gene	neurosporene
pAC-LYCipi	Contains crtE, crtB, crtI, and idi genes of *Erwinia herbicola* (*Pantoea agglomerans*) Eho10	lycopene
pAC-DELTA	Contains crtE, crtB, and crtI genes of *Erwinia herbicola* (*Pantoea agglomerans*) Eho10, together with the lcyE gene of *Arabidopsis thaliana*	δ-carotene
pAC-BETAipi	Contains ctrE, crtB, crtI, crtY, and idi genes of *Erwinia herbicola* (*Pantoea agglomerans*) Eho10	β-carotene
pAC-ZEAXipi	Contains crtE, idi, crtI, crtY, crtB, and crtZ genes of *Erwinia herbicola* (*Pantoea agglomerans*) Eho10	zeaxanthin
pAC-VIOL	Contains crtE, idi, crtI, crtY, crtB, and crtZ genes of *Erwinia herbicola* (*Pantoea agglomerans*) Eho10 and ZEP from *Arabidopsis thaliana*	violaxanthin

All the plasmids are low copy bacterial cloning vectors and provide chloramphenicol resistance.

Table 2. Expression vectors and conditions used for *in vivo* characterization of CCDs activities.

Expression vector	Resistance	Induction	References
pGEX-2T	Ampicillin	0.1 mM IPTG	[46,39]
pGEX 5X-3	Ampicillin	0.1 mM IPTG	[47,38]
pDEST15	Carbenicillin	0.1% arabinose	[30,38]
pDEST15	Carbenicillin	0.2% arabinose	[34]
pDEST14	Ampicillin	0.1% arabinose	[48]
pDEST14	Ampicillin	0.2% arabinose	[31,33]
pBK-CMV	Kanamycin	1 mM IPTG	[37]
pTWIN1	Ampicillin	0.1 mM IPTG	[40]
pBAD/THIO-TOPO®TA	Ampicillin	0.2% arabinose	[22,49]
pBAD/THIO	Ampicillin	0.2% arabinose	[27,35]
pBAD/TOPO	Ampicillin	0.08% arabinose	[36]
pTHIO-DAN1	Ampicillin	0.2% arabinose	[25]
pGEX-4T1	Ampicillin	0.1 mM IPTG	[42,50]
pGEX-4T1	Ampicillin	0.2 mM IPTG	[51]
pET28	Kanamycin	0.5 mM IPTG	[52]
pRSETA	Ampicillin	1% glucose	[53]

Table 3. Expression vectors and conditions used for *in vitro* characterization of CCDs activities

Expression vector	Resistance	Induction	Reference
pGEX-2T	Ampicillin	0.2 mM IPTG	[46]
pGEX-4T1	Ampicillin	0.2 mM IPTG	[42]
pGEX-4T1	Ampicillin	No induction	[54,50]
pThio-DAN2	Ampicillin	0.2% arabinose	[22,24-26,55]
pBAD/THIO-TOPO®TA	Ampicillin	0.2% arabinose	[49]
pGEX 5X-2	Ampicillin	0.2 mM IPTG	[35,36]
pGEX 5X-3	Ampicillin	0.2 mM IPTG	[47]
pGEX-6P-1	Ampicillin	0.1 mM IPTG	[41]