



🔒 Functional analysis of BoCCDs by color complementation in *Bixa orellana* V.2

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Abstract

To determine that BoCCD1-1 and BoCCD4-3, have enzymatic activity and that they can function *in vitro*, these proteins were expressed in lycopene-accumulating *E. coli* (pACCRT-EIB). ORFs BoCCD1-1 and BoCCD4-3 were successfully cloned in the expression vector pDEST17, and the recombinant proteins were expressed in pACCRT-EIB strains to establish cleaving activity.

Materials

MATERIALS

 pCR™8/GW/TOPO **Invitrogen - Thermo Fisher Catalog #No K2500-20**

 Gateway® pDEST™17 Vector **Invitrogen - Thermo Fisher Catalog #Cat. No. 11803-012**

pCR™8/GW/TOPO vector (Invitrogen No K2500-20)

arabinose 2%

BL21 strains

pACCRT-EIB

pDEST™17 vector (Invitrogen No. 11803-012)

UHPLC-ESI-QTOF-MS/MS

BioTek Cytation™3 multi-mode detection system

FTIR (Fourier Transform infrared absorption spectra)

- 1 To study the expression of BoCCD1-1 and BoCCD4-1 and BoCCD4-3 in *E. coli*, ORFs were amplified using the primer specific and introduced into the cloning vector pCR™8/GW/TOPO (Invitrogen No K2500-20), and then to the expression vector pDEST™17 by LR reaction, Gateway Recombination Cloning Technology (Invitrogen No. 11803-012).

Recombinant colonies were co-transformed to BL21 strains using the lycopene-producing plasmid (pACCRT-EIB). Positive colonies were selected using antibiotics ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml) and grown in a Luria-Bertani (LB) liquid medium before being introduced into 2 % arabinose (W/V). The product was isolated as reported by Sinkin et al. 2004 with few modifications and the transformed colony was minced and inoculated to 5 ml pre-inoculum LB medium containing antibiotics chloramphenicol (50 µg/ml) and ampicillin (100 µg/ml) which was left agitating overnight at 37 °C.

- 2 The next day all the pre-inoculum was transferred to  100 mL of LB culture medium and left shaking overnight at room temperature of 24 °C. Expression was induced using 0.2 % arabinose following the handbook instructions (Cat. No. 11801-016, Invitrogen).
- 3 Subsequently, the culture was transferred to  50 mL Falcon tubes and centrifuged at 9000 rpm for  00:10:00 ; supernatant was discarded, and the pellet was re-suspended in  5 mL of chloroform/ethanol (2:1) and centrifuged until color was eliminated, leaving white cells; later vortex was applied for 10 min and the supernatant was collected. Once the supernatant was obtained, it was evaporated with nitrogen gas and stored at  -80 °C until further use in analysis by UHPLC-ESI-QTOF-MS/MS.
- 4 The spectrum of absorbance and fluorometry was performed using the BioTek Cytation™3 multi-mode detection system. Parameter of absorbance spectrum were 230 at 800 nm, step 10 nm with reading speed of 100 msec. The fluorescence spectrum was 420, 460 and 490 nm.
- 5 The mass profiles of pACCRT-EIB, pDEST17BoCCD1-1, and pDEST17BoCCD4-3 extracts were analyzed with Waters XEVO-G2XSQTOF equipment; two types of injections were performed: a) direct injection with 5 µl/min flow, with a range of mass detection of 100-1000 m/z and average reading 1 min ESI+ Source; b) injection by UHPLC, the compounds were separated on a C18 1.7 µm, ACQUITY UPLC BEH column (1.7 µm) at 0.4 µL/min of flow, column temperature 25 °C, and 0.4 µL/min flow, with a mobile phase consisting of ACN:Isoprop:MeOH (75:10:15:1, v/v) (solvent A) and ACN: formic acid (99:1, v/v)

(Solvent B) in gradient from A:B 25:75 to 15:85 in 3 min; then from 15:85 to 10:90 in 5 min; and finally from 10:90 to 25:75 in 20 min. The column eluate was split to allow only 0.45 mL/min to enter the ESI interface. The mass spectra were acquired with a scan range from 100 to 1000 m/z; the MS parameters were set as follows: ESI source in positive ion mode; capillary voltage: 32 kV, sampling cone: 42, source offset 80 and N₂ gas flows: cone gas 50 L/h, desolvation gas 5000 MS/MS cone voltage 35V, energy collision 25V dry gas.

- 6 Identification of functional groups of the products of the expression of pDEST17BoCCD1-1 and pDEST17BoCCD4-3 in *E. coli* was performed with the Cary 630 FTIR spectrometer (portable) (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a five-bounce zinc selenite ATR accessory, in the spectral range 4000 to 500 cm⁻¹.