Plant Gene 5 (2016) 13–21

Contents lists available at ScienceDirect

Plant Gene

journal homepage: www.elsevier.com/locate/plantgene

Isolation and characterization of plastid terminal oxidase gene from carrot and its relation to carotenoid accumulation

M. Doroteia Campos [a], Catarina Campos [a], Hélia G. Cardoso [a,*], Philipp W. Simon [b], Manuela Oliveira [c], Amaia Nogales [a], Birgit Arnholdt-Schmitt [a,*]

[a] EU Marie Curie Chair, ICAAM — Instituto de Ciências Agrárias e Ambientais Mediterrânicas, IIFA-Instituto de Formação e Investigação Avançada, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7002-554 Évora, Portugal

[b] U.S. Department of Agriculture, Agricultural Research Service, Vegetable Crops Research Unit and Department of Horticulture, University of Wisconsin–Madison, 1575 Linden Drive, Madison, WI 53706, USA

[c] Departamento de Matemática, Escola de Ciências e Tecnologia, Centro de Investigação em Matemática e Aplicações, IIFA - Instituto de Investigação e Formação Avançada, Universidade de Évora, Ap. 94, 7002-554 Évora, Portugal

ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form 27 October 2015
Accepted 29 October 2015
Available online 5 November 2015

Keywords:
DcPTOX
Daucus carota
Gene isolation
Expression analysis
Carotenoid biosynthesis
Development

ABSTRACT

Carrot (*Daucus carota* L.) is a biennial plant that accumulates considerable amounts of carotenoid pigments in the storage root. To better understand the molecular mechanisms for carotenoid accumulation in developing storage roots, plastid terminal oxidase (*PTOX*) cDNA was isolated and selected for reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Present in photosynthetic species, PTOX is a plastid-located, nucleus encoded plastoquinone (PQ)-O_2 oxidoreductase (plastioquinol oxidase). The enzyme is known to play a role as a cofactor for phytoene desaturase, and consequently plays a key role in the carotenoid biosynthesis pathway. A single *PTOX* gene was identified (*DcPTOX*) in carrot. *DcPTOX* encodes a putative protein with 366 amino acids that contains the typical structural features of PTOXs from higher plants. The expression of *DcPTOX* was analysed during the development of white, yellow, orange, red, and purple carrot roots, along with five genes known to be involved in the carotenoid biosynthesis pathway, *PSY2*, *PDS*, *ZDS1*, *LCYB1*, and *LCYE*. Expression analysis revealed the presence of *DcPTOX* transcripts in all cultivars, and an increase of transcripts during the time course of the experiment, with differential expression among cultivars in early stages of root growth. Our results demonstrated that *DcPTOX* showed a similar profile to that of other carotenoid biosynthetic genes with high correlation to all of them. The preponderant role of *PSY* in the biosynthesis of carotenoid pigments was also confirmed.

1. Introduction

The plastid terminal oxidase (PTOX) is a nucleus encoded plastid-located plastoquinone (PQ)-O_2 oxidoreductase (plastoquinol oxidase) that occurs widely in photosynthetic species, including algae and higher plants (Cournac et al., 2000; Carol and Kuntz, 2001; Archibald et al., 2003; Kuntz, 2004). *PTOX* is present in some eukaryotic algae as a small multigene family, composed by two members (*PTOX1* and *PTOX2*). In higher plants *PTOX* appears as a single gene (Wang et al., 2009a; Houille-Vernes et al., 2011), which is involved in chlororespiration, chromorespiration and carotenoid biosynthesis (Josse et al., 2000; Carol and Kuntz, 2001; Joet et al., 2002; Aluru and Rodermel, 2004; Kuntz, 2004; Shahbazi et al., 2007). PTOX is the terminal oxidase of chlororespiration, regulating the redox state of the PQ pool (Peltier and Cournac, 2002; Aluru and Rodermel, 2004). It transfers the excess of electrons to O_2, in order to maintain the relative redox balance in the photosynthetic electron transport chain (ETC), which reduces oxidative damage (McDonald et al., 2011). PTOX is considered to play a role in minimizing the generation of reactive oxygen species (ROS) when induced under environmental stresses (McDonald et al., 2011). Sun and Wen (2011) further suggested a protective function with stress-induced inhibition of photosynthetic ETC.

Carotenoid pigments are important compounds in human health because they function as vitamin A precursors and have antioxidant properties as well. Carrot (*Daucus carota* L.) is a biennial plant that provides an important source of carotenoids in the human diets in its storage root. Carotenoids play essential biological roles in plants and the genes coding for enzymes in the carotenoid pathway have already been subject of intensive studies in many species. However, the molecular regulation of carotenoid accumulation in the storage root of carrot has not been extensively explored.

* Corresponding authors at: EU Marie Curie Chair, ICAAM — Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7002-554 Évora, Portugal.
E-mail addresses: hcardoso@uevora.pt (H.G. Cardoso), eu_chair@uevora.pt (B. Arnholdt-Schmitt).

http://dx.doi.org/10.1016/j.plgene.2015.10.005

Recent reports point to PTOX as a key enzyme in the carotenoid biosynthesis pathway. Using a transgenic approach, Carol and Kuntz (2001) showed that the lack of PTOX blocks carotenoid synthesis. PTOX absence gives rise to the *immutans* phenotype in *Arabidopsis thaliana* and to the *ghost* phenotype in *Solanum lycopersicum* (also known as *Lycopersicon esculetum*) (Carol et al., 1999; Wu et al., 1999; Josse et al., 2000; Carol and Kuntz, 2001; Rodermel, 2001; Aluru et al., 2006). These phenotypes are characterized by variegated leaves with green and bleached sectors and additionally – in *S. lycopersicum* – by a yellow-orange ripe fruit. In *immutans*, the variegated phenotype might thus be due to a block in the desaturation of phytoene in the carotenoid biosynthetic pathway, as a result of insufficient oxidized PQ, which is needed as an electron acceptor for this reaction (Wu et al., 1999; Carol and Kuntz, 2001), leading to photobleaching of green tissues. PTOX has also a preponderant role in carotenoid biosynthesis in fruit chromoplasts (Josse et al., 2000), as observed in the yellow-orange *S. lycopersicum* fruit, which is characterized by reduced carotenoid content (Barr et al., 2004). In *S. lycopersicum*, a dual role for PTOX in efficient carotenoid desaturation as well as in chlororespiration in green tissues is referred by Shahbazi et al. (2007). However, *PTOX* transcript levels and carotenoid accumulation are not correlated in all tissues and organs (Aluru et al., 2001).

Protein sequence analysis shows that PTOX shares sequence similarity with the stress-inducible mitochondrial alternative oxidase (AOX) in a number of plant species (Berthold and Stenmark, 2003; Carol et al., 1999; Wu et al., 1999). As with AOX proteins, PTOX sequence analysis reveals the existence of several conserved domains, such as iron-binding residues (McDonald et al., 2011). In both enzymes the sequences exhibit the iron-binding motifs at their C-terminus, typical of Type II di-iron carboxylate proteins (Carol and Kuntz, 2001).

In the present work, the *PTOX* gene was isolated from *D. carota* (*DcPTOX*) and its expression was investigated in relation to carotenoid content in the developing storage root of white, yellow, orange, red, and purple cultivars. These results were compared with the expression of five genes encoding carotenoid biosynthesis enzymes. To our knowledge this is the first report about the isolation of *PTOX* in *D. carota* and the analysis of its expression.

2. Material and methods

2.1. Plant materials

For *DcPTOX* gene isolation, seeds of *D. carota* L. cv. Rotin were germinated *in vitro* in pots containing MS solid medium (Murashige and Skoog, 1962) maintained under controlled conditions (25 ± 1 °C at 16 h photoperiod: 34 μmol m^{-2} s^{-1} light intensity, provided by day light Philips lamps). cDNA from a pool of eight week-old *in vitro* grown seedlings was used for gene identification, while cDNA from a single plant was used for complete gene isolation.

To study the involvement of *DcPTOX* in carotenoid accumulation, an experiment with five cultivars representing a wide range of pigmented carrot material was performed under greenhouse conditions. The cultivars were white (711-1), yellow (207-1), red (203-1), and purple (purple phloem with yellow xylem) (699-1) (cultivated carrot breeding stocks developed by the USDA carrot breeding programme), and the orange coloured cv. Rotin. Seeds of each cultivar were sown in three pots with a total of 10 plants per pot. Four to six plants of each cultivar were collected arbitrarily (biological replicates) at different time points: 5, 7, 9 and 13 weeks post sowing (wps). Samples consisted of complete roots (for samples collected at 5 and 7 wps) or pieces from the upper third root part (for samples collected at 9 and 13 wps). The appearance of the roots during the time course of the experiment can be observed in Fig. S1.

All collected samples were ground to a fine powder using liquid nitrogen and stored at −80 °C until further analysis.

2.2. Total RNA isolation

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), with on-column digestion of DNA with the RNase-Free DNase Set (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The quantification of RNA and the evaluation of its purity were determined in a NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA integrity was evaluated by denaturing gel electrophoresis and visualized using a Gene Flash Bio Imaging system (Syngene, Cambridge, UK) after staining in an EtBr solution (2 ng mL^{-1}).

2.3. Identification of DcPTOX and rapid amplification of the cDNA ends (RACE)

Single strand cDNA was produced with *RevertAid™HMinus M-MuLV Reverse* enzyme (Fermentas, Ontario, Canada) using the oligo (dT) primer *VIAL 8* (Roche, Mannheim, Germany) (Table S1), according to the manufacturer's instruction. The degenerate primer pair (*ptox_613fw* and *ptox_1023rv*, see sequence in Table S1) was designed by choosing the two most conserved regions on an alignment performed with plant *PTOX* gene sequences available at NCBI data bases (National Center for Biotechnology Information, Bethesda, USA) (not shown) and was used for *D. carota PTOX* (*DcPTOX*) gene identification. PCR was performed with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) using 1 μL of cDNA as template and 0.2 μM of each primer. Based on this initial *DcPTOX* cDNA partial sequence obtained by the degenerated primer pair, new primers were designed to conduct Rapid Amplification of the cDNA Ends (RACE) to isolate the 5′ and 3' end of the gene. To determine the 5′-end of *DcPTOX* gene a cDNA library of *D. carota* cv. Marktgaertner M853 (kindly provided by Dr. Bettina Linke, Humboldt University of Berlin, Germany) cloned into a Lambda gt22a phage vector (Invitrogen, Karlsruhe, Germany) was generated (Linke et al., 2003). 5′ RACE-PCR was carried out using 1 μL of cloned library as template and the vector specific forward primer *P6* (Table S1) combined with a gene-specific reverse primer (*DcPTOX_24Rv*, annealing at 58 °C for 30 s and extension at 72 °C for 60 s, see Table S1). For *DcPTOX* 3′-end isolation, 3′ RACE-PCR was conducted using the reverse primer *VIAL 9* (Roche, Mannheim, Germany) in combination with a gene-specific forward primer (*DcPTOX_364Fw*, annealing at 58 °C for 30 s and extension at 72 °C for 60 s, see Table S1). One μL of a 1:10 cDNA dilution of the first strand PCR product was used as template for amplification. RACE-PCRs were performed with Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, England) and 0.2 μM of each primer. For complete gene isolation one gene-specific primer set (*DcPTOX_13Fw* and *DcPTOX_1183Rv*, annealing at 55 °C for 15 s and extension at 72 °C for 60 s, Table S1) was designed based on the 5′ and 3′-UTR sequences previously isolated with RACE-PCRs. One μL of a 1:10 cDNA dilution from a single plant was used as template.

All PCRs were performed in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). PCR products were separated in 1.4% agarose gel, stained with EtBr (2 ng mL^{-1}) and subsequently visualized, on a Gene Flash Bio Imaging system (Syngene, Cambridge, UK). Fragments showing the expected size were purified from agarose gel using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, England) according to the manufacturer's protocol. For cloning, PCR fragments were inserted onto a pGem®-T Easy vector (Promega, Madison, WI, USA) and used to transform *Escherichia coli* JM109 (Promega Madison, WI, USA) competent cells. Plasmid DNA was extracted from putative recombinant clones (Birnboim and Doly, 1979) and confirmed by restriction enzyme analysis using *EcoR*I (Fermentas, Ontario, Canada). Sense and antisense strands were sequenced (Macrogen company: www.macrogen.com) in selected recombinant clones using T7 and SP6 primers (Promega, Madison, WI, USA).