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

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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.

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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.

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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 escunletum*) (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 *Eco*RI (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).

2.4. Bioinformatic analysis of the full-length DcPTOX cDNA and putative protein sequence

Sequence homology was explored at the NCBI database using the BLAST algorithm (Karlin and Altschul, 1993) (http://www.ncbi.nlm.nih.gov/) (BLASTn). To edit *DcPTOX* sequence data, SeqMan and EditSeq software (LASERGENE 7, GATC Biotech, Konstanz) were used. Phylogenetic studies included the PTOX proteins from a group of 21 eudicot and 5 monocot plant species retrieved from the genomic database freely available Plaza (Plaza 3.0: http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_monocots/ and http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/), and were based on a ClustalW Multiple alignment made in BioEdit software (Hall, 1999); the alignment was bootstrapped with 1000 replicates by the Neighbour-Joining (NJ) method using the MEGA 4 software. For protein sequence comparison, a ClustalW Multiple alignment was performed using the CLC Main Workbench 6.7.1 software (CLC bio). TargetP 1.1 software (Emanuelsson et al., 2000) (http://www.cbs.dtu.dk/services/TargetP/) was used to predict the chloroplast targeting sequence cleavage site.

2.5. Reverse transcription quantitative real-time PCR (RT-qPCR)

DNase-treated total RNA (1 μg) was reverse transcribed with the random decamer primers provided by the RETROscript® kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. RT-qPCR was used to investigate the response of *DcPTOX* to carotenoid accumulation in five cultivars of carrot with different root colours. Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, USA). The genes considered for normalization were: elongation factor-1alpha (*EF-1A*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and the ribosomal RNA 5.8S (*5.8S rRNA*) (previous selected by Campos et al., 2015). The target genes selected for RT-qPCR were *DcPTOX*, and the carotenoid biosynthetic genes: phytoene synthase 2 (*PSY2*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), lycopene β-cyclase 1(*LCYB1*), and lycopene ε-cyclase (*LCYE*). Primer sequences and amplicon sizes are shown in Table S2.

Quantification of gene expression was performed by RT-qPCR with SYBR Green q-PCR Master Mix (Fermentas, Ontario, Canada) on a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA). 18 μL reaction volume containing 5 μL of first-strand cDNA (previously diluted 1:10) and 560 nM of each specific primer was used for expression analysis. The identity of each amplicon was confirmed by Sanger sequencing and specificity of qPCR reactions was evaluated by melting curve analysis. Efficiencies were calculated using a 4-point standard curves from a 4-fold dilution series (1:1–1:125) (run in triplicate) of pooled cDNA. RT-qPCR was conducted for 40 cycles, each consisting of 15 s at 95 °C followed by 1 min at 60 °C. To analyse dissociation curve profiles, an additional step at 95 °C during 15 s was added, followed by a constant increase of temperature between 60 and 95 °C. All samples were run in duplicate. Minus reverse transcriptase and no template controls were included to assess contaminations. Cq values were acquired for each sample with the Applied Biosystems 7500 software (Applied Biosystems, Foster City, CA, USA) with a fluorescence threshold arbitrarily set at 1.

Evaluation of expression stability of reference genes and selection of the most appropriate combination of genes to be used for data normalization were done using the statistical application *geNorm* (Vandesompele et al., 2002). Error bars shown in RT-qPCR data represent the standard error of the means of 4 to 6 biological replicates per time point.

2.6. Statistics

The gene expression values were transformed by using a $\log_e$ transformation to ensure a normal distribution and homogeneity of variance (tested by Shapiro–Wilk and the Levene's tests for normality and homocedasticity respectively). A two-way analysis of variance (ANOVA) and a Tukey's honestly significant difference (HSD) for unequal N (Spjotvoll and Stoline, 1973) were used to test for significant differences in gene expression between time points and/or cultivars. Statistical analyses were performed by using software SAS commercial software (Statistical Analysis System) v.9.2. In all the analyses we considered significant a p-value < 0.05.

The correlation between transcript levels of *PTOX* and the other studied genes in all cultivars and time points was evaluated by a parametric correlation test using SPSS v. 16.0.

3. Results

3.1. Characterization of DcPTOX cDNA

Specific primers allowed the isolation of the complete *PTOX* gene of *D. carota* L. cv. Rotin (*DcPTOX*). *DcPTOX* full-length cDNA sequence of 1338 bp (Fig. 1) includes a continuous open reading frame (ORF) of 1098 bp, which encodes a putative polypeptide of 366 amino acid residues, with a predicted molecular weight (mol wt.) of 41.73 kDa and a hypothetical isoelectric point (pI) of 5.57. The homologous identity score performed in NCBI with the deduced amino acid residue sequence shows that DcPTOX shares high degree of similarity with PTOX from other plant species such as C*apsicum annuum* (72% identity, 93% coverage), *S. lycopersicum* (71% identity, 98% coverage) and *Coffea canephora* (70% identity, 94% coverage). In most of the analysed sequences, *PTOX* appears as a single gene. Nevertheless, in five species (*Cucumis melo*, *Eucalyptus grandis*, *Glycine max*, *Populus trichocarpa* and *Zea mays*) two *PTOX* genes were identified (results not shown).

A multiple sequence alignment of 10 complete protein-coding regions of several PTOX plant sequences, including DcPTOX was used to highlight similarities and differences in the protein sequences (Fig. 2): DcPTOX revealed structural features usually found in most of the higher plants' PTOX. These perfectly conserved residues are four glutamates and two histidines, also conserved in DcPTOX at the positions: E151, E190, H193, E242, E312, and H315 (position at the alignment sequence, see in Fig. 2). The conservation of 6 cysteines at the C-terminal region was also noted, as in other plant species (C240, C316, C322, C346, C354, C360 in Fig. 2). A conserved 16-amino acid residues insertion, identified by Fu et al. (2005), which corresponds to exon 8 of the higher plant genomic *PTOX* gene sequence was located near de C-terminus (Fig. 2).

PTOXs from the eudicot group can be clearly distinguished from the monocots by specific residues at the positions E86, M204 and W218 (Fig. 2). PTOX sequences from monocots (Fig. 2) can be also distinguished from the remaining plants by specific residues, F191, R213, F214, F217, A225, R243, L255, A269, N276 and E345. These results were confirmed in an alignment with a larger group of sequences (results not shown). PTOX encoded peptides from *G. max* (GM09G01130 and GM15G11950) and *Z. mays* (ZM02G01080 and ZM02G01090) were included in the alignment. Few differences can be detected between both *G. max* PTOX sequences, only differing in 5 amino acids (Fig. 2). *Z. mays* presented many amino acids differences between the two PTOX sequences (Fig. 2).

DcPTOX protein was predicted as located in the chloroplast, with the predicted length of the chloroplast transit peptide (cTP) from the beginning of the sequence of 49 amino acids residues (cTP score of 0.988). Prediction of cTP for the sequences used in the alignment of Fig. 2 shows no conservation across species. PTOX sequences from *G. max* (GM09G01130 and GM15G11950) showed a conserved predicted length of the cTP of 36 amino acid residues. *A. thaliana* (AT4G22260) cTP displayed 56 amino acids. The Solanaceae members *S. lycopersicum* (SL11G011990) and *Solanum tuberosum* (ST11G007260) showed a cTP of 65 amino acids. PTOX sequences from *Z. mays*, ZM02G01080 and ZM02G01090, present a cTP of 94 and 46 respectively.

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ATTTTTCGATTTTTAGTCAGCTATGGCGATTTTTACGCTCTCTTCAGCTATCTCTGCAATTTCATCTTCGTCTTCATCGTCTTTTAAGCC 90
                              M  A  I  F  T  L  S  S  A  I  S  A  I  S  S  S  S  S  S  S  F  K  P
TAATATTTCTAATAATTTTTCATCGTTTTCGTGTAATTCGTTGAGATTTAATCCTTTGGTTTTGCCTCAGTCTTGCTCTGCTTCTTCACA 180
 N  I  S  N  N  F  S  S  F  S  C  N  S  L  R  F  N  P  L  V  L  P  Q  S  C  S  A  S  S  H
TCCTTCGTTTTCCCGGAAGCTGTTTAAAGTCCAAGCAAGAATTTTGCAAGAGAATGAGGAGAAGGTAGTTGTGGAGGACTCCTTCCAACC 270
 P  S  F  S  R  K  L  F  K  V  Q  A  R  I  L  Q  E  N  E  E  K  V  V  V  E  D  S  F  Q  P
AAAGAGTTCTCCTGAAGTTGACGGAAGTGGAAGTGATAGGGAGCCACCAGATGGTTCATCCTCCAGTGGCATTGAAAGATGGGTTATAAA 360
 K  S  S  P  E  V  D  G  S  G  S  D  R  E  P  P  D  G  S  S  S  S  G  I  E  R  W  V  I  K
GCTCGAACAATCTATCAACATCTTGCTCACTGATTCAGTGATTAAGATTCTTGACACTTTATACCATGACCGTGACTATGCAAGGTTTTT 450
 L  E  Q  S  I  N  I  L  L  T  D  S  V  I  K  I  L  D  T  L  Y  H  D  R  D  Y  A  R  F  F
TGTTCTAGAAACAATTGCAAGAGTTCCTTATTTCGCTTTTATGTCTGTTTTACATATGTATGAGAGTTTTGGTTGGTGGAGAAGGGCTGA 540
 V  L  E  T  I  A  R  V  P  Y  F  A  F  M  S  V  L  H  M  Y  E  S  F  G  W  W  R  R  A  D
CTATCTGAAAGTCCATTTTGCCGAGAGCTGGAATGAAATGCACCATCTGCTCATAATGGAAGAACTGGGAGGAAATGCTTGGTGGTTTGA 630
 Y  L  K  V  H  F  A  E  S  W  N  E  M  H  H  L  L  I  M  E  E  L  G  G  N  A  W  W  F  D
TCGCTTTCTTTCGCAACACATAGCAGTATTCTATTATTTTATGGCAGCATTCATGTACTTGTTGAGTCCAAGAATGGCGTATCATTTCTC 720
 R  F  L  S  Q  H  I  A  V  F  Y  Y  F  M  A  A  F  M  Y  L  L  S  P  R  M  A  Y  H  F  S
TGAATGTGTGGAACATCATGCCTTTGAAACATACGACAAATTTATCAACGCAAAAGGAGAGGATTTGAAAAAGTTGCCTGCATCAAAGGT 810
 E  C  V  E  H  H  A  F  E  T  Y  D  K  F  I  N  A  K  G  E  D  L  K  K  L  P  A  S  K  V
TGCTATAAAATACTACACTGAAGGTGACATGTACTTATTTGATGAGTTTCAAACTTCCAGAGCTCCTAACACACGAAGACCAGTGATAGA 900
 A  I  K  Y  Y  T  E  G  D  M  Y  L  F  D  E  F  Q  T  S  R  A  P  N  T  R  R  P  V  I  D
TAACTTGTATGATGTATTCGTAAACATCAGAGATGATGAAGCAGAACATTGCAAGACAATGAAGGCTTGTCAAACTCCTGGAAACCTCCG 990
 N  L  Y  D  V  F  V  N  I  R  D  D  E  A  E  H  C  K  T  M  K  A  C  Q  T  P  G  N  L  R
TTCTCCTCACTCAGGCTTTGACAATGCTTTTGAAGATGATGCAGGATGTATATTGCCTGATACAAGTTGTGAAGGTATCGTAGACTGTAT 1080
 S  P  H  S  G  F  D  N  A  F  E  D  D  A  G  C  I  L  P  D  T  S  C  E  G  I  V  D  C  I
AAAGAAATCTGTCACACGTGACACGTCCTCAGTCAATTGAGAAAGAGATATTAGGCAAGTAGGATGATAATATACCATATCATAGGAACA 1170
 K  K  S  V  T  R  D  T  S  S  V  N  *
TATACAGTAGGAAAATAGTAATACAAAATAGAATTAAATCATCACAAAATGGTATTCTTAAACGAATATCTAGTTGTAACGAAAAGGAGG 1260
CTTGTAATTTTTAGTTTGTTGTGTACAACATTGTAACAGTCATATGCATATCGGATTCATATAAAAAAAAAAAAAAAA 1338
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Fig. 1. Nucleotide and deduced amino acid sequences of cDNA encoding *Daucus carota* L. cv. Rotin plastid terminal oxidase, *DcPTOX*. * indicates stop codon (GenBank: EU331420).

To determine the relationship between DcPTOX and PTOX from other plant species, a NJ tree was constructed (Fig. 3), using the identified PTOX sequences. In plants, PTOX can be separated into two different groups: one group includes all eudicots sequences and another the monocots (Fig. 3). A clade that comprises members of Brassicaceae (AL7G19990, AT4G22260, BR01G12490, CRU_007G18140, TP7G20380) can also be clearly identified within the eudicots.

3.2. DcPTOX gene expression and its relation to carotenoid biosynthesis

To examine whether carotenoid accumulation during carrot root development and differences in carotenoid composition between carrot cultivars could be related to the expression of *PTOX*, transcript levels of *DcPTOX* and five carotenoid biosynthetic genes were analysed. *EF-1A* and *GAPDH* were selected to normalize target gene expression. Carotenoids analysis confirmed that the dominant carotenoids that accumulate in the analysed carrot roots vary according to root colour and increased during carrot developmental age (results not shown).

RT-qPCR analysis performed from carrot tap roots during secondary root growth showed that all the analysed genes were expressed between the 5th and 13th week of plant growth in the five cultivars, including the white carrot, where very little or no carotenoid pigments were detected (Fig. 4).

Target gene transcript accumulation in the different cultivars throughout the investigated period can be observed in Fig. 4 (for differences during development for each genotype see uppercase letters). Similar to other carotenoid biosynthetic genes (Fig. 4B, C, D) *DcPTOX* presented an increasing transcript level during the time course of the experiment, with significant ($p < 0.05$) differences during early root development (5–9 wps) (approximately 3-fold for white, yellow and purple; 2-fold for red and 4-fold for orange, Fig. 4A). This is consistent with the accumulation of total carotenoids in coloured cultivars during root development. Carotenoid accumulation and expression levels of most genes seemed to be less correlated during the late rapid carotenoid accumulation stage (9–13 wps). For most of the studied genes, and for all cultivars, transcript levels were stable over this period, with *LCYB1* being the only exception. Although the white cultivar does not contain carotenoids (see below), expression of *DcPTOX*, *PDS*, *ZDS1* and *LCYB1* increased globally throughout the investigated period. *PSY2* remained constantly low in this cultivar (Fig. 4) with a slight increase in the period between 5 and 7 wps.

Differences in transcript levels between cultivars in each time point can also be seen in Fig. 4 (see lowercase letters). At 7, 9 and 13 wps, *PSY2* presented differential expression between the white and the other cultivars, with a maximum of 11.6-fold difference from the orange cultivar, at 9 wps. *LCYE* was low in the white cultivar in all studied time points, and significantly different from all cultivars at 13 wps. *PDS*, *PTOX* and *ZDS* presented a similar profile, with some differences between different root colours only detected at 7 wps.

Transcript levels of *DcPTOX* correlated with the expression of *PSY2* (Pearson coefficient: 0.747, $p < 0.001$), *PDS* (Pearson coefficient: 0.810, $p < 0.001$), *ZDS* (Pearson coefficient: 0.721, $p < 0.001$), *LCYB1* (Pearson coefficient: 0.791, $p < 0.001$) and *LCYE* (Pearson coefficient: 0.631, $p < 0.001$).

4. Discussion

4.1. DcPTOX sequence analysis

A single gene encoding PTOX was identified in *D. carota* L. cv. Rotin (*DcPTOX*), similarly to the reported in higher plants such as

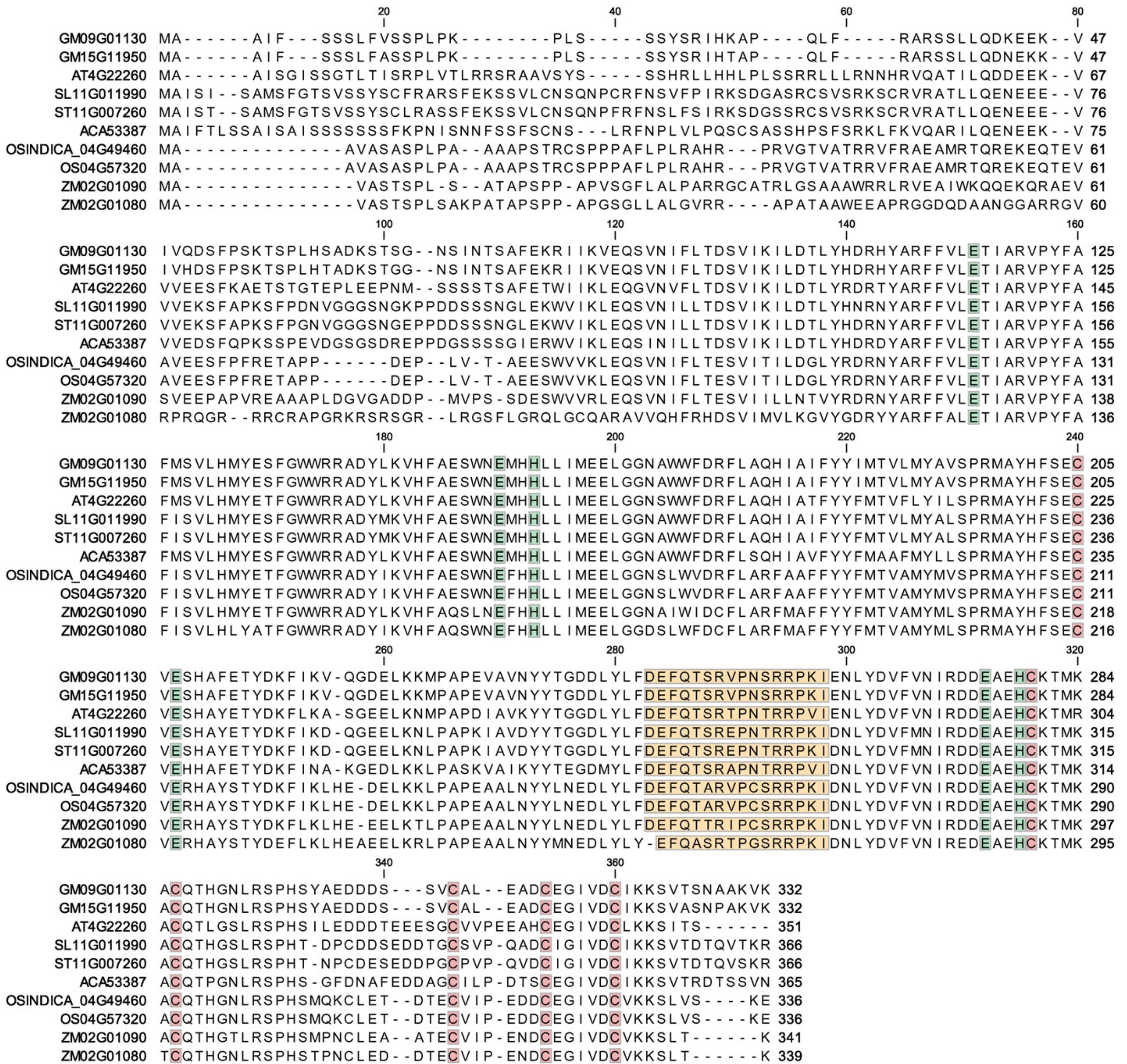

Fig. 2. Multiple alignment of the deduced amino acid sequence from *Daucus carota* L. cv. Rotin PTOX (DcPTOX) and 9 other plant PTOX sequences. The alignment was performed using CLC Main Workbench 6.7.1 software. Six conserved cysteines identified by Josse et al. (2000) are indicated in red. The amino acids required for iron binding are indicated in green (Berthold et al., 2000). The location of the exon 8 identified by Fu et al. (2005) is boxed in yellow. The predicted amino acid sequences used for comparison were DcPTOX (GenBank: ACA53387) and other PTOX sequences from Plaza databases as follows: GM09G01130 and GM15G11950, *Glycine max*; AT4G22260, *Arabidopsis thaliana*; SL11G011990, *Solanum lycopersicum*; ST11G007260, *Solanum tuberosum*; OSINDICA_04G49460, *Oryza satica indica*; OS04G57320, *Oryza sativa japonica*; ZM02G01090 and ZM02G01080, *Zea mays*.

S. lycopersicum, *C. annuum* and *Arabidopsis* (Scolnik et al., 1987; Carol et al., 1999; Wu et al., 1999; Josse et al., 2000), as well as in several phylogenetic distant plant species included in this analysis like *Brachypodium distachyon* and *Sorghum bicolor*. Nevertheless, it was possible to identify, by analysis of sequences available from all genome sequencing projects, the existence of two *PTOX* genes in some plant species (including mono and dicot plant species) (Fig. 2, detailed information in Campos et al. in prep) e.g. in *C. melo*, *E. grandis*, *G. max*, *P. trichocarpa* and *Z. mays*. The deduced amino acid sequence of *DcPTOX* shared high sequence similarity with PTOXs from other higher plants, including the conserved iron binding residues described by several authors (Josse et al., 2000; Finnegan et al., 2003; Fu et al., 2009; McDonald et al., 2011). PTOX is a member of the non-heme diiron carboxylate (DOX) protein family. The DOX domain is composed of a four-helix bundle that provides six ligands for binding the diiron center (Andersson and Nordlund, 1999; Berthold et al., 2000). Fu et al. (2005) demonstrated by using in vitro and *in planta* site-directed mutagenesis, that the iron ligands of PTOX are essential for activity and that they do not tolerate changes. These mutagenesis experiments also showed a highly conserved 16 amino acid domain located near the C-terminus, encoded by exon-8, required for PTOX activity and stability. That domain, characteristic of PTOX (McDonald et al., 2003; Fu et al., 2005, 2009; McDonald et al., 2011), can exceptionally be absent. That is the case of *A. thaliana*, in which an allele of *IM* gene (*PTOX* gene for *A. thaliana*) is characterized by the lack of exon-8 (Aluru et al., 2006). That mutation does not affect gene regulation at the transcriptional

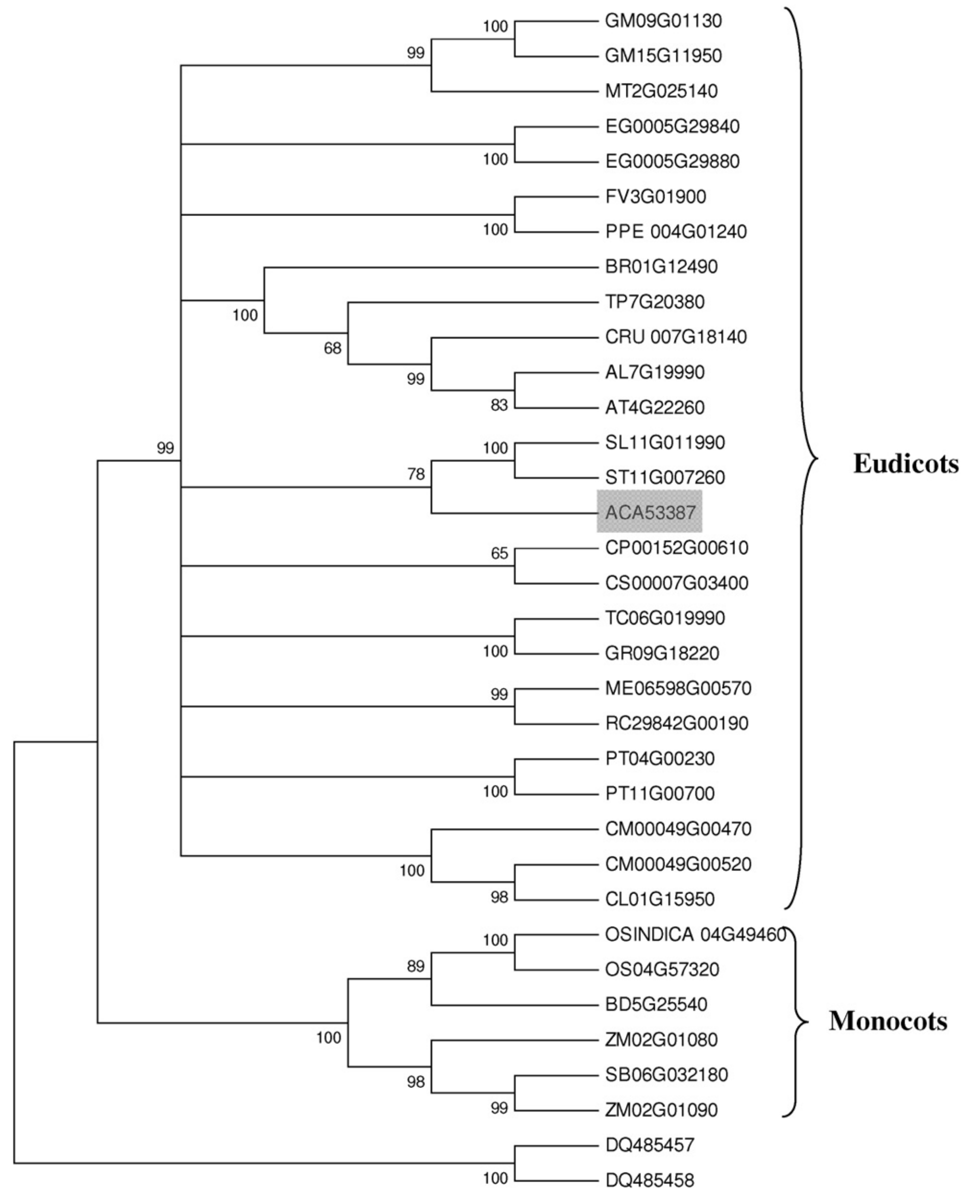

Fig. 3. Neighbour-Joining (NJ) tree showing the relationships among 32 deduced PTOX sequences from plants. The shaded area indicates DcPTOX. The NJ tree was obtained using the complete PTOX peptide sequences. The alignments were bootstrapped with 1000 replicates by the NJ method using the MEGA 4 software. PTOX sequences from the green algae *Haematococcus pluvialis* (GenBank: DQ485457 and DQ485458), were used as outgroups. The predicted amino acid sequences used for comparison were *Daucus carota* PTOX (GenBank: ACA53387) and other PTOX sequences from Plaza databases as follows: GM09G01130 and GM15G11950, *Glycine max*; MT2G025140, *Medicago truncatula*; EG0005G29840 and EG0005G29880, *Eucalyptus grandis*; FV3G01900, *Fragaria vesca*; PPE_004G01240, *Prunus persica*; BR01G12490, *Brassica rapa*; TP7G20380, *Thellunginela parvula*; CRU_007G18140, *Capsella rubella*; AL7G19990, *Arabidopsis lyrata*; AT4G22260, *Arabidopsis thaliana*; SL11G011990, *Solanum lycopersicum*; ST11G007260, *Solanum tuberosum*; CP00152G00610, *Carica papaya*; CS00007G03400, *Citrus sinensis*; TC0006G22260, *Theobroma cacao*; GR09G18220, *Gossypium raimondii*; ME06598G00570, *Manihot esculenta*; RC29842G00190, *Ricinus communis*; PT04G00260 and PT11G02180, *Populus trichocarpa*; CM00049G00470 and CM00049G00520, *Cucumis melo*; CL01G15950, *Citrullus lanatus*; OSINDICA_04G49460, *Oryza satica indica*; OS04G57320, *Oryza sativa japonica*; BD5G25540, *Brachypodium distachyon*; ZM02G01090 and ZM02G01080, *Zea mays*; SB06G032180, *Sorghum bicolor*.

level but has a strong role on post-transcriptional gene regulation; normal levels of mRNA are produced, but no IM protein was accumulated. Six previously reported conserved cysteines on the C-terminal region (Josse et al., 2000) were also identified in DcPTOX.

PTOX chloroplast localization was confirmed by *in organelle* import assays in *A. thaliana* (Wu et al., 1999; Carol et al., 1999). Like most of the precursor proteins targeted to chloroplasts, PTOX has a N-terminal cTP, and its length influences the efficiency of translocation (Bionda et al., 2010). In PTOXs of higher plant, no conservation across species was found when a prediction of cTP was done using bioinformatics tools. The biological reason for those differences remains unknown. Nevertheless, there are other examples of no conservation of TP across plant species. It is the case of the mitochondrial AOX where the predicted length of the mitochondrial targeting sequence cleavage site is highly variable, not only across species but also within the same plant species across proteins encoded by different members of the *AOX* gene family (Campos et al., 2009).

4.2. DcPTOX, carrot development and carotenoid accumulation

Carotenoids are common pigments, and their composition and content in plants are important indexes to evaluate the nutritional and commercial values. For a better understanding of the involvement of *PTOX* on the carrot carotenoid biosynthesis pathway, an experiment involving five cultivars with variable root colours and different developmental stages was performed. *DcPTOX* transcripts

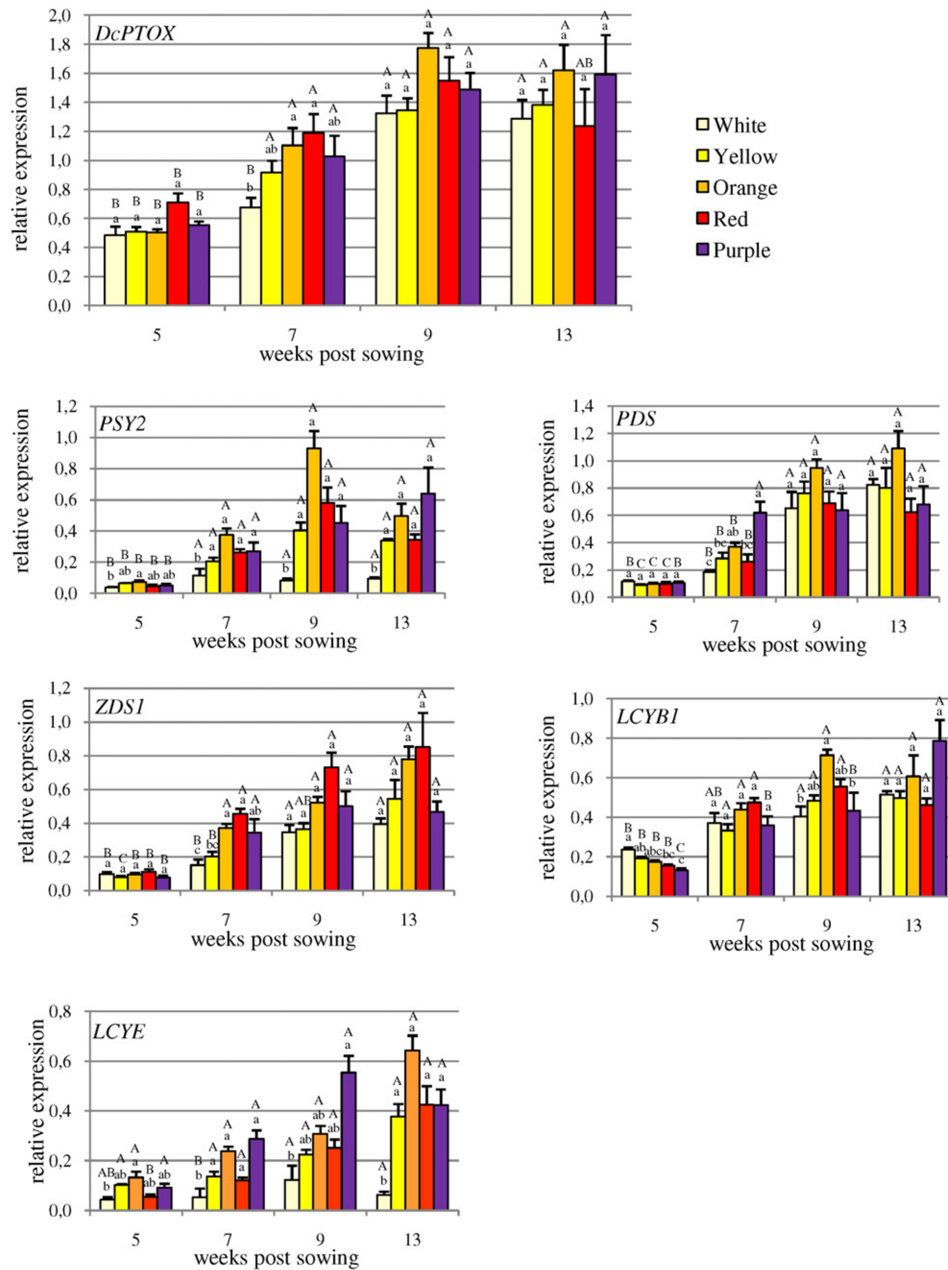

Fig. 4. Transcript accumulation of *DcPTOX* and five carotenoid biosynthetic genes during carrot root development in five different carrot cultivars, growing under greenhouse conditions. Transcript levels were determined by RT-qPCR. In each harvest time point, 4–6 biological replicates were considered per cultivar. Error bars indicate the standard error of the mean. Different superscript letters indicate significant differences between the stages of development for each cultivar (capital letters), or between cultivars within each time point (small letters).

were ubiquitously detected between the 5th and 13th week of plant growth, for all cultivars. Beside *DcPTOX*, five other genes directly involved in carotenoid biosynthesis pathway, with transcripts previously detected in carrot roots, were selected (Just et al., 2007; Clotault et al., 2008; Fuentes et al., 2012). *DcPTOX* presented a similar profile to these genes, and showed high correlation to all regarding transcript accumulation, especially with *PDS*. PTOX is known to play a role as a cofactor for PDS and is consequently a key enzyme in carotenoid biosynthesis. In the carotenoid biosynthetic pathway, the coloured carotenoids are synthesized within plastids from phytoene, a non-coloured precursor that results from two geranylgeranyl diphosphate (GGPP) molecules, catalysed by PSY. Desaturation of phytoene by the sequential activity of the enzymes PDS and ZDS (Carol and Kuntz, 2001; Simkin et al., 2008) results in the production of lycopene, a substrate for the formation of both α- and β-carotene. Catalytic activity of PDS and ZDS has been shown to require several redox components including PQ and O_2. As a PQ/O_2 oxidoreductase, plant PTOX has been regarded as an important co-factor of carotenoid biosynthesis by transferring the electrons derived from PDS and ZDS steps to O_2 via the PQ pool (Carol and Kuntz, 2001; Aluru and Rodermel, 2004). PTOX is responsible for oxidizing PQH_2 and making PQ available for reduction allowing the process to continue (Josse et al., 2000; McDonald et al., 2011). Lack of PTOX will block carotenoid synthesis at the PDS step due to over reduction of the PQ pool (Carol and Kuntz, 2001). Due to its position in metabolism, PDS activity could serve to regulate both the carotenoid and chlorophyll biosynthetic pathways (McDonald et al., 2011). In tomato, the loss of PTOX function in the *ghost* mutant leads to carotenoid accumulation defects in both leaves (chloroplasts) and fruit (chromoplasts), and was shown to be a major cofactor involved in phytoene desaturation in both photosynthetic and non-photosynthetic tissues (Josse et al., 2000).

Carrot root colour is a result of various pigments that serve as intermediate products in the carotenoid pathway. Different root colours are the result of differences in carotenoid composition. The orange colour is due to α- and β-carotene, whereas the red colour is due mostly to an accumulation of lycopene. The purple carrots contain anthocyanins, and the yellow colour in carrot roots is due to xanthophylls, downstream of β-carotene. White-coloured roots are low in total carotenoids. However, in our experiment, a poor link was detected between carotenoid accumulation and the relative transcript abundance of *DcPTOX*, as well as other carotenoid biosynthetic genes. In fact, only in the early stages of root growth, differences on *DcPTOX* expression were detected among cultivars. However, differential expression of *PSY2* was detected when comparing the carrot storage root tissue of the white and the other cultivars. These results are in agreement with Maass et al. (2009), where they suggest that *PSY* is the major driver for accumulation of carotenoids, and with Santos et al. (2005) that refer an important role of *PSY* in regulating carotenoid accumulation in a carrot population segregating for white, yellow and orange root colour. From the analysed carotenoid biosynthetic genes in different carrot storage root colours (*PTOX* not included), also Bowman et al. (2014) observed differential expression only on *PSY* genes, suggesting its key role in the biosynthesis of carotenoid pigments. These authors hypothesised that differential expression of one or more genes in that pathway may account for, or at least contribute to, the large differential accumulation of carotenoids observed by comparing white and orange carrots. Evidence of carotenoid gene expression in non-pigmented carrot root (without carotenoid accumulation), with a similar profile of several carotenoid genes of coloured roots (including *DcPTOX*) was observed in our study, as well as by several other authors (Bowman et al., 2014; Clotault et al., 2008). Some mechanisms were already suggested to explain this apparent paradox such as the existence of non-functional alleles, tissue-specific isoforms, impaired enzyme activity, or increased carotenoid degradation in white carrots, but no direct experimental evidence supporting any of them has been presented (Rodrígues-Concepción and Stange, 2013; Clotault et al., 2008). Nevertheless, the identification of post-transcriptional mechanisms with influence on carotenoid accumulation might contribute to explain this poor correlation (Ruiz-Sola and Rodríguez-Concepción, 2012). As an example of this post-transcriptional mechanisms, we can refer to the orange curds of the cauliflower (*Brassica oleracea* var. *botrytis*) *Orange* variety, in which chromoplast-like plastids with inclusions of membranous compartments develop due to a mutation in a gene that results in the accumulation of much higher carotenoid (β-carotene) levels compared to uncoloured varieties, without changes in the expression of carotenoid biosynthetic genes (Li et al., 2001). Although the biological role of this mutation is still unclear, these studies illustrate how carotenoid accumulation can be boosted by triggering the synthesis of a plastid deposition sink to store carotenoids (Giuliano and Diretto, 2007; Li and Van Eck, 2007). Nevertheless, the existence of miRNAs considered as "master regulators" due to their role on the regulation of gene expression, acting at transcriptional and post-transcriptional levels (Wu et al. 2010), with a crucial role in plant growth and development (Bartel, 2004; Mallory and Vaucheret, 2006; Voinnet, 2009) should be taken in account. There are also reports elucidating the molecular biology of different miRNAs related to fruit ripening, softening, ethylene biosynthesis and the signal transduction of different phytohormones (Wang et al., 2009b; Elitzur et al., 2010; Bapat et al., 2010; Cara and Giovannoni, 2008). The involvement of miRNAs on the control of carotenoid accumulation was already described in sweet orange related with lycopene accumulation (Xu et al., 2010). More recently, Khaldun et al. (2015) report fruit-specificity of some miRNAs from which some were already described as regulators of genes involved in the carotenoid biosynthesis pathway.

From our results, we can suggest an additional explanation for the increase of *PTOX* transcripts in the carrot white cultivar along the experiment, which is based on the association of *DcPTOX* not only with carotenoid biosynthesis, but also with root development and growth. However, future analysis on DcPTOX protein content and activity are initiated that aims a better understanding of the involvement of DcPTOX on both processes.

As a complement to gene expression, *DcPTOX* genomic sequence analysis, with the putative identification of single nucleotide polymorphisms (SNPs) and InDels (insertion and deletions) in all parts of the gene (exons, introns, promoter regions and UTRs) will be important to determine if these features are associated with storage root pigmentation. DNA sequence analysis in carotenoid biosynthesis genes has already been associated with accumulation of various carotenoid pigments. In maize, the natural variation of DNA sequence of the carotenoid gene *LCYE* has been found to control the flux of carotenoid metabolism towards either α-carotene or β-carotene accumulation, and can be used as a source of genetic variation for maize biofortification (Harjes et al., 2008). Sequence variability in *DcPTOX* could also contribute to the diversity in carotenoid accumulation in *D. carota*. Association of these polymorphisms will help bridge the gap between genomics and phenomics.

In conclusion, our results demonstrated that *DcPTOX* expression has a similar profile to that of other carotenoid biosynthetic genes, with high correlation to all, suggesting an involvement of *DcPTOX* in carotenoid biosynthesis pathway during carrot storage root development. Our results also reaffirm previous experiments regarding the preponderant role of *PSY* in the biosynthesis of carotenoid pigments. We also propose that *DcPTOX* might be associated not only with carotenoid biosynthesis, but also with development and growth of carrot tap root.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plgene.2015.10.005.

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