Daucus carota as a novel model to evaluate the effect of light on carotenogenic gene expression

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ABSTRACT

Carotenoids are synthesized in prokaryotic and eukaryotic organisms. In plants and algae, these lipophilic molecules possess antioxidant properties acting as reactive oxygen species scavengers and exert functional roles in hormone synthesis, photosynthesis, photomorphogenesis and in photoprotection. During the past decade almost all carotenogenic genes have been identified as a result of molecular, genetic and biochemical approaches utilizing *Arabidopsis thaliana* as the model system. Studies carried out in leaves and fruits of *A. thaliana* and tomato determined that light regulates carotenoid biosynthesis preferentially through the modulation of carotenogenic gene transcription. In this work we showed for the first time that light induces accumulation of psy1, pds and zds2 transcripts in leaves of *Daucus carota* (carrot), a novel plant model. In addition, modified roots of carrots exposed to light accumulate zds1, whereas the pds gene is highly repressed, suggesting that some carotenogenic genes, which are expressed in roots, are regulated by light. Additionally, light negatively regulates the development of the modified carrot root in a reversible manner. Therefore, this suggests that light affects normal growth and carotenogenic gene expression in the modified root of carrot plants. The molecular insight gained into the light-regulated expression of carotenoid genes in this and other model systems will facilitate our understanding of the regulation of carotenoid biosynthesis to improve the prospects for the metabolic engineering of carotenoid production in plants.

Key terms: carotenoid biosynthesis, carrot, gene expression, light regulation.

INTRODUCTION

Carotenoids are lipid-soluble molecules of 40 carbons that are synthesized in a wide variety of photosynthetic and non photosynthetic organisms including plants, algae, some fungi and bacteria but not animals. In plants and algae, carotenoids are synthesized in the plastids, such as chloroplasts and chromoplasts. In chloroplasts, these pigments are localized and accumulate in the thylakoid membranes (Cunningham and Gantt, 1998), acting as accessory pigments in the Light Harvesting Complex (LHC), during photosynthesis (Britton, 1995). Carotenoids are also synthesized and accumulated in lipid bodies or in crystalline structures inside chromoplasts, plastids that accumulate pigments in flowers, fruits and reserve or modifies roots (Vishnevetsky *et al*., 1999). In flowers and fruits, the presence of these pigmented molecules serves to attract pollinators and seed dispersal agents by the intense yellow, orange and red colors that they provide to these organs (Grotewold, 2006).

In addition, carotenoids are precursors for the biosynthesis of the plant hormone abscisic acid (Crozier *et al*., 2000), protect plant cells from photo-oxidative damage by quenching singlet oxygen produced from chlorophyll triplet in the reaction center of photosystem II (Takano *et al*., 2005; Telfer, 2005) and exert a photoprotective role during excessive light incidence through thermal dissipation by means of the xanthophyll cycle, protecting the plant from photo-oxidative damage.

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Carotenoids are not synthesized by animals. Therefore, they must be ingested in the diet for the subsequent synthesis of related molecules such as vitamin A, retinal and retinoic acid, which play essential roles in nutrition, vision and cellular differentiation, respectively (Krinsky *et al*., 1994). Furthermore, these molecules have also been shown to delay the aging process due to their antioxidant properties (Mordi, 1993; Bartley and Scolnik, 1995). At the same time, oxidative damage associated with several pathologies, including aging (Esterbauer *et al*., 1992), carcinogenesis (Breimer, 1990) and degenerative processes in humans, among others, can be resisted by ingestion of carotenoids (Snodderly, 1995; Mayne, 1996, Rao and Rao, 2007). Birds, fish and crustaceans utilize carotenoids for pigmentation and nutritional purposes. For example, the cetocarotenoid astaxanthin is responsible for the orange color of salmon meat and lobster shells (reviewed in Grotewold, 2006). Carotenoids also have agronomic and commercial importance in several ornamental plants, in the cosmetic and food industries (Klaui and Bauernfeind, 1981) and are employed as poultry and fish feed additives (reviewed in Bjerkeng, 2000).

Until the sixties, research in this area had been centered on the biosynthetic route of carotenoids (Cunningham and Gantt, 1998). Subsequently, almost all genes termed carotenogenic genes that codify for enzymes involved in the metabolism of carotenoids in diverse plant species, algae, fungi and bacteria have been identified and characterized (Hirschberg *et al*., 1997; Cunningham and Gantt 1998; Cunningham, 2002; Naik *et al*., 2003; Lodato *et al*., 2004).

In plants, carotenogenic genes are encoded in the nuclear genome and the synthesized proteins are targeted as preproteins to the plastids, where they are post-translationally processed. Carotenoid biosynthesis in chloroplasts begins with the synthesis of the isoprenoid isopentenyl pyrophosphate (IPP; Fig. 1), through the non-mevalonate route (Schwender *et al*., 1996; Lichtenthaler *et al*., 1997), by condensing D-glyceraldehyde 3-phosphate with pyruvate, forming 1-deoxy-D-xylulose-5-phosphate (DOXP) (Shanker *et al*., 2003; Rohmer, 1999), a reaction catalyzed by DOXP-synthase (DXS). In subsequent steps and catalyzed by DOXP reductoisomerase (DXR), hydroxymethylbutenyl diphosphate (HBMPP) synthase (HDS) and HBMPP reductase (HDR), DOXP is transformed into IPP (Lichtenthaler, 1999). IPP molecules synthesized in the plastids are then isomerized to the allylic isomer, dimethylallyl pyrophosphate (DMAPP) by means of IPP isomerase (IPI). Subsequently, DMAPP condenses with three molecules of IPP to generate a molecule of 20 carbons named geranylgeranyl pyrophosphate (GGPP), in a process involving GGPP synthase (GGPPS). The formation of the symmetrical 40-carbon phytoene from two molecules of GGPP is catalyzed by phytoene synthase (PSY) in a two-step reaction. Phytoene biosynthesis is the first reaction specifically related to the carotenoid biosynthesis pathway. The biosynthesis of carotenoids continues with the desaturation of the colorless phytoene to produce the pink-colored trans-lycopene (Fig.1). These reactions are catalyzed by phytoene desaturase (PDS), forming ζ-carotene, ζ-carotene desaturase (ZDS), which synthesizes pro-lycopene (7, 9, 9', 7'-tetra-cis-lycopene) and carotene isomerase (CRTISO), which transforms pro-lycopene into lycopene (all-trans-lycopene) in plants (Isaacson *et al*., 2002; Park *et al*., 2002). In leaves, the activity of CRTISO is substituted by light that photoisomerizes ζ-carotene, neurosporene, and prolycopene (Isaacson *et al*., 2002). Subsequently, lycopene is transformed into different bicyclic molecules. It has been observed that in plants and algae two enzymes participate in the cyclization of lycopene (Cunningham *et al*., 1996; Cunningham *et al*., 2007), lycopene-β-cyclase (LCYB), which converts lycopene into γ-carotene and subsequently to β-carotene and lycopene-ε-cyclase (LCYE) that cyclizes one end of the lycopene molecule with an ε-ring (δ-carotene), whereas the other ring is formed by LCYB,

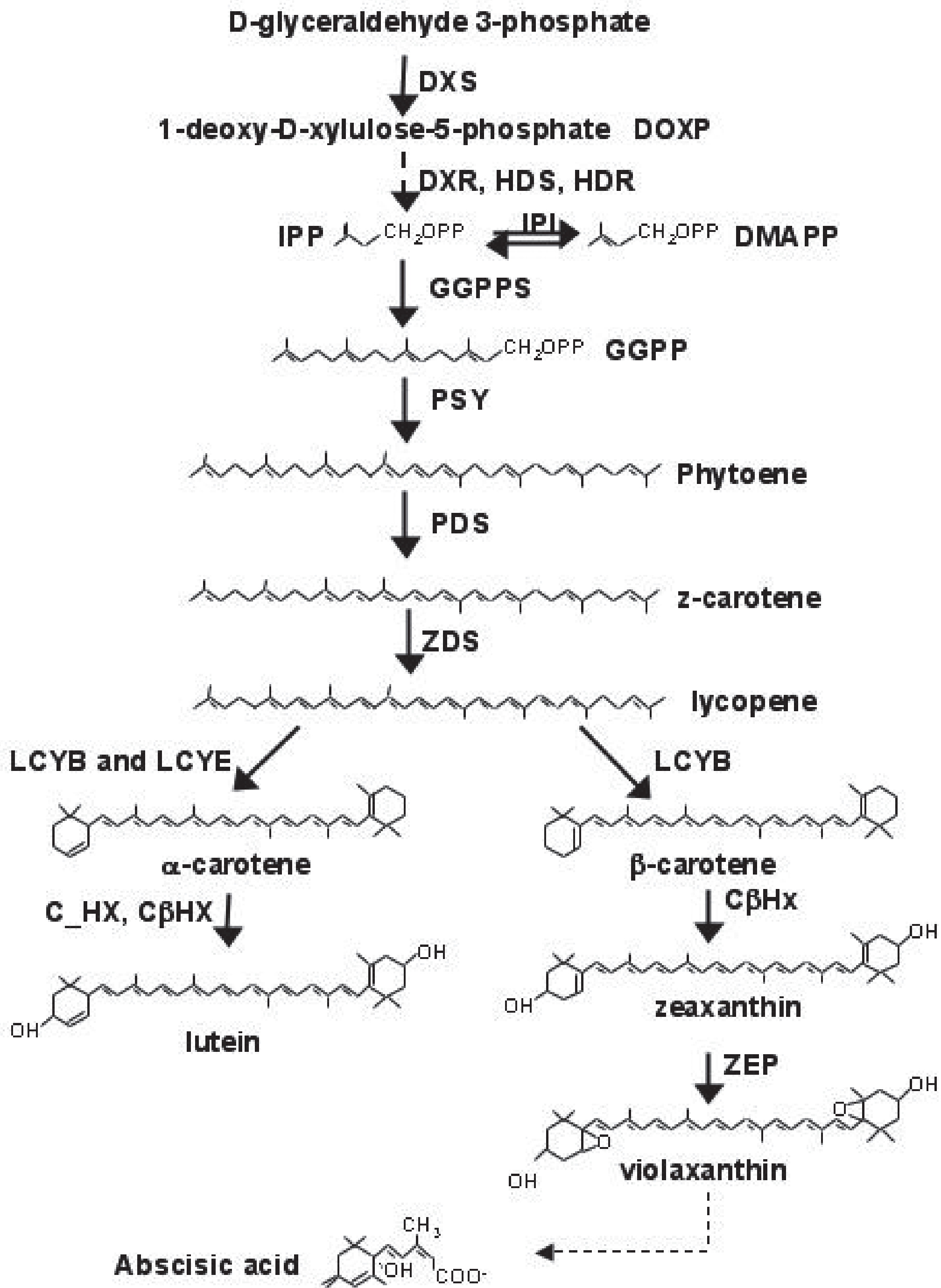

Figure 1: Schematic representation of carotenoid synthesis in plants. The isopentenyl pyrophosphate (IPP) is synthesized in plastids through the non-mevalonate route, and begins with the synthesis of DOXP catalyzed by DOXP synthase (DXS). The other enzymes that participate in the biosynthesis of carotenoids and abscisic acid are: isopentenyl pyrophosphate synthase (IPI), geranylgeranyl pyrophosphate synthase (GGPPS), phytoene synthase (PSY), phytoene desaturase (PDS), z-carotene desaturase (ZDS), carotene isomerase (CRTISO), lycopene ε cyclase (LCYE), lycopene β cyclase (LCYB), β-carotene hydroxylase (CβHx), ε-carotene hydroxylase (CεHx) and zeaxanthin epoxidase (ZEP). The name and structure of the synthesized carotenes and xanthophylls are included.

thus producing α-carotene (Cunningham *et al*., 1996). The β-carotene synthesized is utilized as substrate for the enzyme β-carotene hydroxylase (CβHx) to produce zeaxanthin, while the hydroxylation of α-carotene by the ε-carotene hydroxylase (CεHx) and CβHx results in the formation of lutein. Finally, abscisic acid is synthesized in the cytoplasm via a series of reactions subsequent to the epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) (Cunningham and Gantt, 1998; Cunningham, 2002; Naik *et al*., 2003).

The regulation of carotenoid biosynthesis has been studied in photosynthetic organs (leaves) and in non-photosynthetic organs (fruits, flowers, tubers and seeds) of traditional plant models, such as *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco) and *Solanum lycopersicon* (tomato) (Römer and Fraser, 2005; Howitt and Pogson, 2006). Almost all of these studies show that carotenogenic genes are expressed in photosynthetic organs exposed to different light qualities, during the transition of etioplasts to chloroplasts (de-etiolation) (Römer and Fraser, 2005; Bramley, 2002). During these processes, carotenogenic gene expression is mostly regulated at the transcriptional level mediated by photoreceptors, such as the family of phytochromes (PHYA-PHYE), cryptochromes (CRY) and phototropins (Simkin *et al*., 2003; Woitsch and Römer, 2003; Briggs and Olney, 2001; Scheppens *et al*., 2004, Franklin *et al*., 2005; Briggs *et al*., 2007).

Light also affects carotenoid biosynthesis in a number of species during fruit ripening and flower development (Zhu *et al*., 2002, 2003; Giovanonni, 2004; Phillips *et al*., 2004; Kishimoto and Ohmiya, 2006). During tomato fruit ripening, expression of dxs, hdr, pds and psy1 is co-ordinately upregulated, whilst at the same time the expression of lycβ and lcyε decreases (Fraser *et al*., 1994; Pecker *et al*., 1996; Ronen *et al*., 1999; Lois *et al*., 2000; Botella-Pavía *et al*., 2004), leading to an accumulation of lycopene in chromoplasts of ripe fruits (Pecker *et al*., 1996). The psy gene is upregulated during fruit development and ripening (Fraser *et al*., 1999, Giuliano *et al*., 1993) and during flower development (Zhu *et al*., 2002, Zhu *et al*., 2003). In tomato, two distantly related genes, psy1 and psy2, code for phytoene synthase, and the former was found to be transcriptionally activated only in petals and in ripening of tomato fruits (Welsch *et al*., 2000; Giorio *et al*., 2008). Psy2 is expressed in all plant organs, preferentially in tomato leaves and petals (Giorio *et al*., 2008), but in green or ripe fruits it is only expressed at low levels (Bartley and Scolnik, 1993; Giorio *et al*., 2008).

Carotenogenic genes are not only expressed in leaves and fruits. In potato, β-carotene and lutein are synthesized (Nesterenko and Sink, 2003) whilst carotenoids are also present in amyloplasts of cereal seeds such as maize and wheat (Panfili *et al*., 2004, see Howitt and Pogson 2006 for review). Both potatoes and cereals accumulate low levels of carotenoids in the dark (Nesterenko and Sink, 2003), in contrast to the highly pigmented modified root of carrots.

Daucus carota L. (carrot, 2n=18) is a biennal plant whose orange reserve or modified root is consumed worldwide due to its high levels of α-carotene and β-carotene (8 mg/g dry weight, Fraser, 2004). The carotenoid composition of a typical orange colored carrot contains predominantly β-carotene (45-80%) and α-carotene that together constitute up to 95% of total carotenoids (Simon and Wolf 1987; Baranska *et al*., 2006). The major physiological function of the carrot root is as a reserve of assimilates for the production of a flowering stem after appropriate stimuli (Hole 1996). Young carrot roots are pale and start to accumulate carotenoids after the first month of growth, levels of which usually peak after three months, shortly before secondary growth is completed (Suslow et al., 1999).

The complete cDNA sequences of some carrot carotenogenic genes, such as isopentenyl pyrophosphate isomerase (ipi, DQ192183), phytoene synthase 1 and 2 (psy1, DQ192186; psy2 DQ192187), z-carotene desaturase 1 and 2 (zds1,

DQ222430; zds2, DQ192189), phytoene desaturase (pds, DQ222429), lycopene β-cyclase (lcyb, DQ192190), lycopene ε-cyclase (lyce, DQ192192) and capsantine capsorubine synthase (ccs: DQ192191), were annotated recently at the NCBI database (Just *et al*. 2007). The kinetics of the transcript accumulation of some of these genes correlates to total carotenoid composition during the development of modified roots grown in the dark (Clotault *et al*., 2008). However, to date, the influence of light on carotenoid gene expression in the leaves and modified root of carrots has not been examined. In this report, we show that this environmental factor differentially affects the accumulation of transcripts of several carotenoid genes and alters the morphology and development of modified roots grown in the presence or absence of light.

MATERIALS AND METHODS

Plant Material

Seeds of commercially-acquired carrot (*Daucus carota L.*) cultivar "Nantaise improved 3" were sown hydroponically and cultivated for 4, 8 and 12 weeks with a 16 h photoperiod illuminated with white fluorescent light (450 μmol m^{-2} s^{-1}) at 20-23ºC. Leaves and modified roots of 12-week-old carrot plantlets were harvested and utilized in real time RT-PCR analysis. For light treatments of carrot roots, the upper segment was exposed to white fluorescent light (450 μmol m^{-2} s^{-1} in a 16 h photoperiod) for 12 weeks, leaving the lower segment to grow in darkness. The sampled roots are depicted in Fig. 2 and 4. For dark treatment of carrot leaves, leaves of 12-week-old carrot plants were protected from light for 48 h with aluminium paper to reduce the light level to 0,06 μmol m^{-2} s^{-1}. Leaves and modified root pieces subjected to light and dark treatment from three carrot plants were pooled, frozen in liquid nitrogen, and powdered to isolate total RNA. Each experiment was performed in triplicate.

Reverse transcription and quantitative RT-PCR

Total RNA was extracted from frozen powder using RNAsolv (Omega Biotec, USA). Genomic DNA traces were eliminated by a 20 min RNase-free DNase I treatment at 37 ºC followed by addition of 25 mM EDTA-DEPC and incubation at 75ºC for 5 min to inactivate the enzyme. For cDNA synthesis, 1 μg of DNA-free RNA was incubated at 70ºC for 5 min with 1mM of oligo dT, then cooled for 5 min on ice. Four μl of Impron II 5x buffer, 0.5 mM each dNTP, 20U of RNase Inhibitor, and 1 μl of Impron II reverse transcriptase (Promega) were added to the RNAs, which were then incubated at 25 ºC for 5 min and at 42 ºC for 60 min. To inactivate reverse transcriptase, the reaction was incubated at 70 ºC for 15 min. The cDNA was then ready for real time RT-PCR, which was performed with the LightCycler system (Stratagene), using SYBR Green I double strand DNA binding dye. Specific primers targeting carotenoid biosynthesis genes (psy1, psy2, pds, zds1 and zds2; Table 1) were designed from the 5' UTR of each gene on the basis of published cDNA sequences (Just et al., 2007). Specific primers were also designed to amplify ubiquitin, the housekeeping gene used as the reference gene. The absence of amplification from genomic DNA in cDNA samples was tested by comparison of PCR products obtained from cDNA and from RNA templates to ubiquitin primers. These analyses confirmed the absence of genomic DNA in all cDNA samples. The amplification of the fragments was carried out in a total volume of 25 μl containing 140 nM of the sense and anti-sense primers, 12.5 μl Brillant SYBR® Green® QPCR Master Mix (Stratagene), 0.375 μl ROX and 2 μl of cDNA, prepared as described above. The following cycling conditions were chosen: melting of the cDNA at 95 ºC for 8 min, amplification with 40 cycles with a denaturation step at 94 ºC for 30 s, annealing at 52 ºC for 40 s, and a final elongation at 72 ºC for 30 s. A reamplifying step at the end of the process was also included: 94ºC for 30s, 50ºC for 30 s and

72ºC for 30 s. Fluorescence data was collected after each extension step. Fluorescence was analyzed using LightCycler Analysis Software. The crossing point for each reaction was determined using the Second Derivative Maximum algorithm and manual baseline adjustment. Gene expression levels were calibrated to the average value of organs analyzed to obtain a Calibrated ΔCt for each gene. Amplification efficiency was determined for each set of primers by amplification of the target from a PCR dilution series and according to the equation:

$$\left(E = \left[10^{\left(-1/\text{slope}\right)} \right] - 1 \right)$$

The value obtained in this equation was used to obtain the ratio between the carotenogenic gene and the ubiquitin gene expression using the following equation (Pfaffl 2001):

$$\text{ratio} = \frac{(E\ \text{carot})^{\Delta CP\ \text{carot}\ (Ct\ \text{ubiq}-Ct\ \text{sample carot})}}{(E\ \text{ubiq})^{\Delta CP\ \text{ref}\ (Ct\ \text{ubiq}-Ct\ \text{sample carot})}}$$

Efficiency values for carotenogenic genes and ubiquitin amplification during the standard curve calibration were between 0.75 and 0.96, and r2 values for these curves were over 90%. Ct values for ubiquitin varied by no more than 2 units among all samples analyzed for each real time experiment. Each qRT-PCR reaction was performed with three biological replicates and each sample was analyzed in duplicate (technical replicate). In all cases, the reaction specificities were tested with melt gradient dissociation curves and electrophoresis gels. To test for significant differences in gene expression, T-test 95% were carried out using the General LinearModels option in the statistical software package Graphpad Prism. Two tailed Student t-test, $p < 0.05$ (confidence interval 95%) was used.

RESULTS

Effect of light on carotenogenic gene expression in leaves of carrots

To evaluate the effect of light in photosynthetic organs, carotenogenic gene expression levels were compared in leaves of

TABLE 1

Primers used for real time RT- PCR analysis of carotenogenic genes expression in *D. carota*

Primer	Gene	Accession number	Sequence (5'-3')	Tm (ºC)	Fragment length (bp)
zds1F	zds1	DQ222430	GAATTCCTCGAATCGGACGGC	66	144
zds1R	zds1	DQ222430	CTCGACGCTTGGCCTACTAAT	64	
zds2F	zds2	DQ192189	ACCGGATCAGAATTCCTCGAAT	62	100
zds2R	zds2	DQ222430	TTCTCGTGGTTCAATACAATTATACAATG	76	
pdsF	pds	DQ222429	TCTCCACCTCCTTCATTCAGTCTAA	62	95
pdsR	pds	DQ222429	GCAAAATGAAGTCGCTATGTGTCT	70	
psy1F	psy1	DQ192186	AGTCGATGGAGCATTACCATAATTC	70	95
psy1R	psy1	DQ192186	CTAATGGGTTACAGAGGGTTGTGTTA	74	
psy2F	psy2	DQ192187	GTTCTTAGCTAAACTTCCGTGGG	74	110
psy2R	psy2	DQ192187	GCTGGAGTTAGTGCTACCC	60	
ubiF	ubiquitin	U68751	GCTCGAGGACGGCAGAAC	60	121
ubiR	ubiquitin	U68751	CTTGGGCTTGGTGTAGGTCTTC	68	

12-week old plants, grown in a diurnal light regime harvested in the light period, with leaves of the same plant subjected to darkness for 2 days. Transcript levels of pds and zds2 were significantly higher in light-treated *D. carota* leaves compared to those maintained in darkness (Fig 2). This result is in agreement with the relative expression analysis of carotenogenic genes performed in *A. thaliana* (von Lintig *et al.*, 1997; Welsch *et al.*, 2000, Botella-Pavía *et al.,* 2004). Psy1 codifies for phytoene synthase, which is involved in the synthesis of phytoene, the branch point for carotenoid biosynthesis. *D. carota* harbors two genes that are proposed to codify for PSY, psy1 and psy2, which share 73% identity at their cDNA sequences. We observed that psy1 transcript levels increased five-fold during light treatments, while psy2 was not affected by the same conditions. Pds and zds2 genes are involved in lycopene biosynthesis and transcript levels of these genes were eleven- and four-fold greater in leaves exposed to light compared to dark-treated leaves, respectively. To date, two sequences (zds1 and zds2), sharing 87% identity, have been annotated in carrot as coding for z-carotene desaturase. In addition, no other plant species has more than one zds gene (Cunningham and Gantt, 1998; Naik *et al.*, 2003). Therefore, their role and relative participation in carotenoid biosynthesis under different conditions and throughout development has yet to be analyzed. We observed that zds2 expression is activated by light treatments, while zds1 transcript accumulation is repressed by light (Fig 2).

Effect of light on modified root development and carotenogenic gene expression

D. carota has a large reserve root that develops in darkness and where high levels of α and β-carotene accumulate (Fraser and Bramley, 2004). We studied the effect of

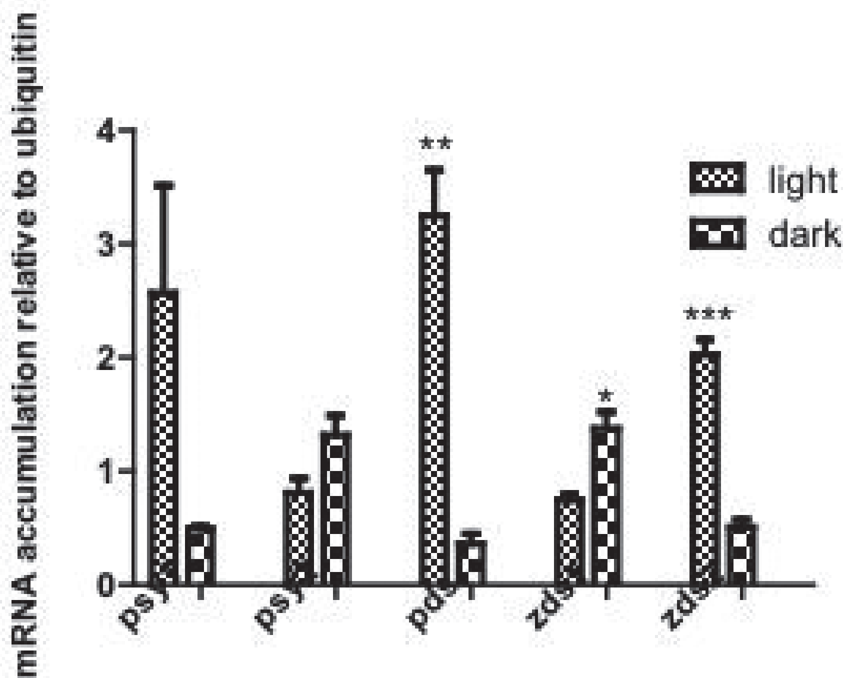

Figure 2: Carotenogenic gene accumulation in leaves of *D. carota* exposed to light or dark conditions. Real time RT-PCR analysis of psy1, psy2, pds, zds1 and zds2 genes during light or dark treatment of leaves in a 12 week- old *D. carota* plant. Results were normalized with ubiquitin and calibrated against the average of each gene's expression for both conditions. Standard deviations (SD) are the result of three independent replicates. Asterisks indicate significant differences in gene expression among treatments. Student t-test, $p < 0.05$.

light on the development of the modified root of carrot. As shown in figure 3, a 4-week-old carrot plant can be segmented into three sections: leaves (L), roots (R) and a root that will become the modified root of the plant (Fig 3A, Rd). When the upper part of the future modified root was exposed to light (Rl), and the lower part (Rd) kept in darkness for 8 weeks, only the latter developed into a normal, expanded orange carrot (Fig 3B). However, when the Rl segment was later protected from light (Rl-d) for a further 4 weeks, this segment developed as a normal modified root (Fig 3C Rl-d and Rd), becoming indistinguishable from the portion of the root grown in continuous darkness (not shown). Therefore, the *D. carota* modified root develops only in darkness (Rd) and light treatments (Rl) inhibit normal development in a reversible manner (Rl-d).

In order to determine the effect of light on carotenogenic gene expression during modified root development, the relative abundance of some carotenogenic genes in roots exposed to light (Rl) and in modified roots grown in normal development conditions (Rd) from a 12-week-old carrot plant was measured by real time RT-PCR (Fig.4).

Psy1, psy2, pds, zds1 and zds2 genes were expressed in both conditions; however, only pds and zds2 expressions were induced in dark grown roots (Rd) relative to the segment of root that was exposed to light (Rl, Fig 4A and B). On the other hand, the relative abundance of zds1 was higher in roots exposed to light (Rl) than in the 12-week-old normal modified root (Rd, Fig 4B), while psy1, psy2 and zds2 transcript levels did not change significantly between the two conditions. These results suggest that the expression of pds and zds1 is regulated by light.

DISCUSSION

In this study, morphological analysis and qRT-PCR were used to determine the effect of light on root development and carotenogenic gene expression in *Daucus carota* L, a novel plant model. This plant produces and accumulates carotenoids in leaves that are exposed to light and in the modified root, grown in darkness. No other plant model synthesizes such amounts of carotenoids in an organ that is not exposed to light. All analyzed carotenogenic genes were expressed in leaves and in modified

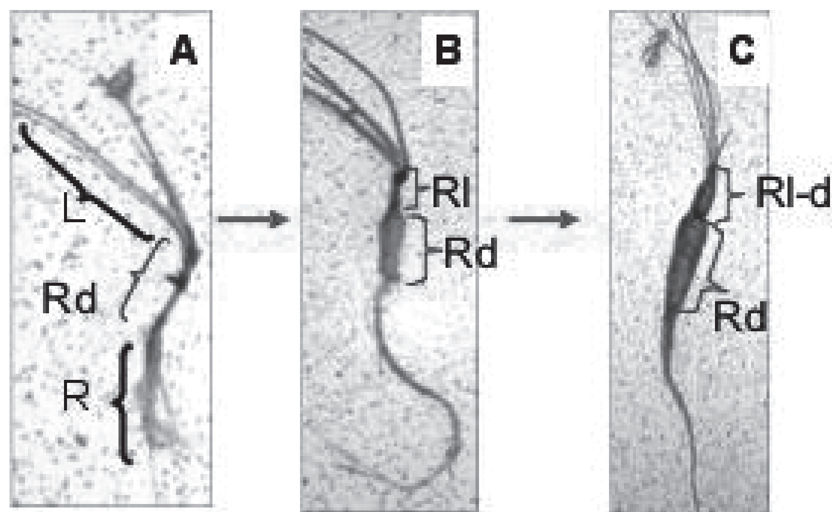

Figure 3: Effect of light on carrot modified root development. A) A 4-week-old *D. carota* plantlet. L: leaves, R: roots, Rd: Root segment that will develop into a modified root. B) An 8-week-old *D. carota* plantlet. Rl: Light-exposed modified root. Rd: Dark-grown modified root. C) A 12-week-old *D. carota* plantlet. Rl-d: Modified root exposed to light for 8 weeks, then covered for 4 weeks. Rd: Normal modified root development. Representative plants are shown (*n*=10).

roots of carrot, although expression levels differed between these organs, suggesting that different regulatory mechanisms leading to carotenoid accumulation are involved in leaves and roots. To our knowledge, these results demonstrate for the first time that the expression profiles of selected carotenogenic genes are differentially influenced in carrots after dark or light treatments. Clotault *et al.*, (2008), analyzed the transcript accumulation of psy1, psy2, pds, lcyb, zds1, zds2, lcye and zep during modified root development in four carrot varieties. It was show that the expression was correlated to the accumulation of carotenoid pigments. However one of them, Blanche, a white variety, does not accumulate carotenoids, but expresses almost all genes during root development (Clotault *et al.*, 2008). Therefore, the expression of carotenogenic genes does not always lead to a functional carotenogenic enzyme, by the presence of non-functional alleles.

We determined that light induces expression of pds and zds2 in leaves and represses the accumulation of zds1 transcripts, whilst psy1 and psy2 were not affected significantly by light or dark treatments. This suggests that carrot pds1, zds1 and zds2 genes respond to light and may have light responsive elements (LRE) in their promoters, as was shown for the psy gene promoter of *A. thaliana,* which has LREs, specifically G-box and G box-like elements that respond to light (Welsch et al., 2003; 2007). The results obtained in carrot leaves are in agreement with the relative expression analysis of carotenogenic genes performed in *A. thaliana* and tomato, in which psy and hdr transcripts increased during the transfer of leaves to light after being in darkness (von Lintig *et al.*, 1997; Welsch *et al.*, 2000, Botella-Pavía *et al.,* 2004). However in *D. carota* leaves, psy2 expression was not affected by light or dark treatments and the induction of psy1 by light was not

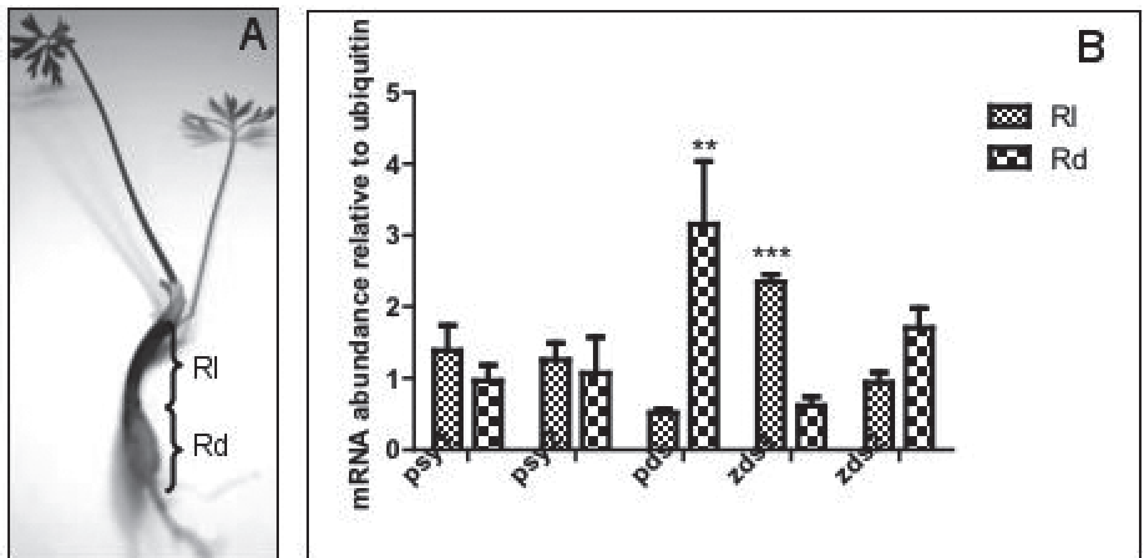

Figure 4: Carotenogenic gene accumulation in roots of *D. carota* exposed to light or dark conditions A) Morphological development of a 12-week-old carrot plantlet. Light: segment of root that was exposed to light for 4 weeks. Dark: segment of root exposed to continuous darkness. B) Relative transcript accumulation of psy1, psy2, pds, zds1 and zds2 was analyzed by means of real time RT-PCR using root samples indicated in A. Standard deviations (SD) are the result of three independent replicates. Expression data were normalized with ubiquitin and calibrated against the average of each gene's expression for both conditions. Asterisks indicate significant differences in gene expression among treatments. Student t-test, $p < 0.05$.

statistically significant in relation to the dark treatment (Fig 2). We are currently isolating and characterizing the promoter of pds and zds1 genes to determine whether LREs are present in the promoter sequences.

During darkness, biosynthesis of carotenoids in leaves is stopped due principally to the very low level of expression of carotenogenic genes. In *C. annum*, psy, pds, zds and lcyb, genes are down regulated under these conditions (Simkin *et al*., 2003) and in *A. thaliana* the psy and hdr genes are active in darkness only at basal levels (Welsch *et al*, 2003, Botella-Pavía *et al.,* 2004). In carrot, psy1, pds and zds2 genes are also expressed at basal levels in leaves treated for 2 days in the dark.

On the other hand, the accumulation of zds1 mRNA was repressed in the presence of light. Carotenoids are synthesized during light exposure, but it was shown that when light intensity increases from 150 to 280 mmol m^{-2} s^{-1} the rate of photo oxidation is higher than the rate of synthesis and carotenoids are destroyed, reaching a basal level (Simkin *et al.,* 2003). The level of expression of some carotenogenic genes are reduced following prolonged illumination at moderate light intensities (Woitsch and Römer, 2003), as was shown for pds transcript accumulation in tomato seedlings and was referred to as inhibition by final product (lycopene) (Corona *et al.,* 1996; Giuliano et al., 1993). This phenomenon could also explain the behavior of carrot zds1.

In roots, pds and zds1 were affected significantly by light treatment, whereas zds2 was not, indicating that pds and zds1 genes might have LREs in the promoters. This observation is in agreement with the results obtained in carrot leaves. In *A. thaliana* leaves, it has been shown that light, through PHYA, plays a role in the transcriptional induction of psy in *A. thaliana* (von Lintig *et al*., 1997), by promoting the binding of the transcription factor HY5 to LREs located in the promoter. It is possible that during modified root development other transcription factors are involved, which are activated by dark conditions and repressed when roots are exposed to light. In this way, pds gene accumulation in modified carrot root could be explained, whereas the expression of zds1 could be associated with a basal level of expression in the modified root through the absence of a specific transcription factor, which is only present in light conditions or could be also associated with repression by final product. Through these results, we can conclude that pds and zds2 genes are important during carotenoid biosynthesis in leaves and in roots, associated directly with normal developmental conditions of each organ.

In contrast to leaves, in fruits, flowers, seeds, root tubers, and reserve roots, carotenoids accumulate in chromoplasts. In these plastids, carotenoids are stored in plastoglobuli, where they are more photo-stable than in chloroplasts (Merzlyak and Solovchenko, 2002). Therefore, photo-oxidation does not affect carotenoid content in these organs, even when they are exposed to light. Our results indicate that light has a negative effect on modified carrot root development, possibly because some transcriptional or growth factors are inhibited, although more extensive studies are needed to investigate this phenomenon. Roots exposed to light did not develop normally (Fig.3) and did not synthesize carotenoids compared to roots developed in darkness (data not shown). Therefore, microarray studies could be a helpful tool for the global comparison of genes that are induced or repressed in roots grown in darkness or exposed to light conditions. To date, many specific factors that are implicated in the transcriptional activation of genes regulated by light, such as carotenogenic genes, have been identified by means of microarray analyses. During initial exposure of *A. thaliana* seedlings to light, many transcription factors (HY5, CCA1, LHY, APRR9, APRR5, HYH, SPA1, PKS1) are early over-expressed (Quail, 2007; Tepperman *et al.,* 2001). However, it has yet to be established whether these transcription factors play a role in the induction of carotenogenic genes.

Conventional studies focused on a specific gene or step in the carotenoid pathway combined with new technologies

permitting an analysis of the entire pathway will be needed to understand the role of light on carotenoid biosynthesis in diverse organisms. Transcriptome analysis will provide insights into regulatory branch points of the pathway, whilst proteomic studies could help to associate the protein/ enzyme component profiles with the carotenoid content in plants. Without doubt, aspects associated with the effect of light on carotenoid biosynthesis regulation will be avenues warranting more intensive research efforts. Research to alter the light-mediated signal transduction machinery would also be an effective approach for modulating chlorophyll and fruit carotenoid content in plants.

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