Abstract

Plant carotenoids are synthesized and accumulated in plastids through a highly regulated pathway. Lycopene β-cyclase (Lcyb) is a key enzyme involved directly in the synthesis of α-carotene and β-carotene through the cyclization of lycopene. Carotenoids are produced in both carrot (Daucus carota) leaves and reserve roots, and high amounts of α-carotene and β-carotene accumulate in the latter. In some plant models, the presence of different isoforms of carotenogenic genes is associated with an organ-specific function. D. carota harbors two Lcyb genes, of which DcLcyb1 is expressed in leaves and storage roots during carrot development, correlating with an increase in carotenoid levels. In this work, we show that DcLcyb1 is localized in the plastid and that it is a functional enzyme, as demonstrated by heterologous complementation in Escherichia coli and over expression and post transcriptional gene silencing in carrot. Transgenic plants with higher or reduced levels of DcLcyb1 had incremented or reduced levels of chlorophyll, total carotenoids and β-carotene in leaves and in the storage roots, respectively. In addition, changes in the expression of DcLcyb1 are accompanied by a modulation in the expression of key endogenous carotenogenic genes. Our results indicate that DcLcyb1 does not possess an organ specific function and modulate carotenoid gene expression and accumulation in carrot leaves and storage roots.

Introduction

Carotenoids are isoprenoid pigments synthesized in plants, algae and some bacteria and yeast. In chloroplasts of photosynthetic organs, they play classical roles in several processes such as light absorption during photosynthesis, photo-protection via energy dissipation and reactive oxygen species (ROS) detoxification. Carotenoids also provide yellow, orange and red colors to fruits and flowers for animal-mediated pollination and seed dispersal. In these organs, carotenoids are synthesized and stored in specialized plastids called chromoplasts. In addition, carotenoids act as precursors of important apocarotenoids such as the growth regulators abscisic acid (ABA, Figure 1A) and strigolactones [1,2,3,4] and of volatile flavour/aroma terpenes [5].

In animals they are fundamental for health and must be taken in through their diets as an essential source of retinoids and vitamin A [6,7]. In humans, carotenoids have been shown to have antioxidant-promoting activities [8,9]. Carotenoids are derived from secondary metabolic processes that take place in plastids, and even though almost all of the nuclear encoded carotenogenic genes have been identified [10,11], there is still limited knowledge concerning the regulation of the carotenoid pathway. Carotenoids are synthesized using IPP derived from the non-mevalonate pathway (MEP) as substrate to produce the common C20 precursor, geranylgeranyl diphosphate (GGPP). Two molecules of GGPP are substrates of phytoene synthase (PSY), the first committed step in carotenoid biosynthesis (Figure 1A). The colorless phytoene is then modified to form the reddish lycopene through sequential desaturations and isomerizations carried out by the enzymes phytoene desaturase (PDS), ζ−carotene desaturase (ZDS), carotenoid isomerase (CRTISO) and ζ-carotene isomerase (ζ-ISO)[12,13]. Hydroxylation of β-carotene and α-carotene results in the synthesis of xanthophylls such as violaxanthin, zeaxanthin and lutein (Figure 1A). Lycopene cyclization is accomplished by lycopene β-cyclase (LCYB), which produces β-carotene (two β-ionone rings) in the presence of NADPH as co-factor, whereas α-carotene is created by LCYB and lycopene ε-cyclase (LCYE) [14,15,16]. The partial pyridine nucleotide binding site is universally present in the lycopene cyclase enzymes, despite the evolutionary divergence between lycopene cyclases of non-photosynthetic bacteria and those of photosynthetic organisms [17,18]. In plants, unlike in bacteria and cyanobacteria, carotenoid enzymes have a signal peptide in the amino terminus for import into the plastids [8,19,20,21,22]. Specifically, the signal peptide in lycopene β-cyclase is found in the first 50–100 amino acids [21,22]. In LCYBs of plants and bacteria, a conserved “dinucleotide binding motif” is also found. In addition, several conserved domains, such as the “Conserved region β-LCY’s”, Cyclase Motif I and II, and a charged region are highly conserved in plants and cyanobacterial LCYBs, and partially conserved in bacterial LCYBs, whereas a domain named “β-cyclase motif” is fully conserved in all plants.
LCYBs. These motifs and regions could be involved in the substrate-enzyme interaction, in membrane association and in catalysis [17,22,23].

In several plant models such as Arabidopsis thaliana [24], Oryza sativa [25] http://rice.plantbiology.msu.edu/), and Zea mays [26,27], LCYB is encoded by a single gene. Nevertheless, two genes encoding lycopene β-cyclase have been found in some plants that accumulate high levels of carotenoids in non-photosynthetic organs, such as fruits and flowers. These genes are differentially expressed in photosynthetic and non-photosynthetic organs. In tomato, SlLcyb is preferentially expressed in green organs, while SlCycb in ripening fruits and flowers [28,29]. Similar organ specific functions of two Lcyb genes have also been described in Capsicum annuum (pepper; [22]), Carica papaya [30,31,32], Crocus sativus [33] and Citrullus lanatus [16].

In order to determine the function of carotenoid genes, several mutants have been described such as Lyb deleterious hemizygous mutants in Oryza sativa, named “pre-harvested stress genotype” (phs-1 and phs-2) and the insertional mutant PS1 in Zea mays [25,27]. These mutants show a phenotype that resembles that of others which are defective in carotenoid synthesis, such as Psy, Pds and Zds mutants, which display leaf bleaching, ROS accumulation and viviparity due to a deficiency in ABA synthesis [25,27,34,35,36,37,38].

In carrots (Daucus carota), β-carotene and α-carotene accumulate to high levels in the storage root, a non-photosynthetic organ [8,39,40], and in the leaves [8,39,41]. Just et al., 2007 [42] identified complete cDNA sequences for most of the carotenogenic genes of D. carota, of which two are candidate sequences to encode for lycopene β-cyclase, DcLcyb1 (DQ192190) and DcLcyb2 (DQ192191). DcLcyb1 is transcriptionally regulated; expression studies showed that LCYB1 transcription increases significantly during storage root development in coordination with an increase in carotenoid and β-carotene levels [39,41]. This expression is affected differentially by light treatments in distinct developmental stages of the root [41].

Here, we compared DcLcyb1 expression in leaves and roots, showing that the expression level is higher in the aerial organs throughout D. carota development. In addition, we analyzed DcLcyb1 function by means of heterologous complementation in E. coli, subcellular localization, post-transcriptional gene silencing and over-expression in D. carota. We conclude that this gene encodes for a functional plastid-targeted LCYB and is required for β-carotene synthesis in both leaves and storage roots of D. carota. Moreover, DcLcyb1 participates in the regulation of endogenous DcPsy1, DcPsy2 and DcLcyb2 carotenogenic genes.

Figure 1. Schematic diagram of carotenoid synthesis in plants. (A) The carotenoid biosynthetic pathway in plants. Enzymatic conversions are shown by arrows with the enzymes involved in each reaction. GGPPS: Geranyl-Geranyl Pyrophosphate Synthase; PSY: Phytoene Synthase; PDS: Phytoene Desaturase; ζ-ISO: ζ-carotene isomerase; ZDS: ζ-carotene Desaturase; CRTISO: Carotenoid Isomerase; Z-ISO: ζ-carotene isomerase, LCYB: Lycopene β-cyclase; LCYE: Lycopene ε-cyclase; B-CHX: β-carotene Hydroxylases; E-CHX: ε-carotene Hydroxylases; ZEP: ZeaxanthinEpoxidase; VDE: Violaxanthin de-epoxidase; NSY: Neoxanthin synthase, CCD: Carotenoid cleavage dioxygenase; NCED: 9-cis-epoxycarotenoid dioxygenase. (B) Lycopene β-cyclase Reaction. The LCYB enzyme transforms one molecule of lycopene into one molecule of β-carotene or α-carotene (together with LCYE) using NADPH as cofactor.

doi:10.1371/journal.pone.0058144.g001
Methodology

Plant Material

Seeds of commercially-acquired carrot (Daucus carota L.) cultivar Nantaise were sown in a mix of soil and vermiculite (2:1) and cultivated in a greenhouse for 4, 8 and 12 weeks with a 16 h long day photoperiod illuminated with cool-white fluorescent light (115 μmol m⁻² s⁻¹) at 20–23°C until molecular analysis were carried out. Hypocotyls of four-week-old in vitro wild-type carrot plantlets cultivated in solidified MS (Murashige and Skoog) medium were harvested and utilized in Agrobacterium-mediated transformation experiments. Transformed carrots were transferred to pots (20×10) and cultivated in the greenhouse, as described above, when they reached 5 cm long.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from a frozen powder of 200 mg of D. carota leaves and storage roots of 4, 8 and 12 week-old plants using RNA solv (Omega Biotec, USA). For cDNA synthesis, 2 μg of total DNA-free RNA was mixed with 1 mM of oligo dT primer and Impron II reverse transcriptase (Promega). Total RNA was extracted from a frozen powder of 200 mg of D. carota leaves and storage roots of 4, 8 and 12 week-old plants using RNA solv (Omega Biotec, USA). For cDNA synthesis, 2 μg of total DNA-free RNA was mixed with 1 mM of oligo dT primer and Impron II reverse transcriptase (Promega). SYBR Green double strand DNA binding dye. Specific primers described in [43] in a Stratagene Mx3000P thermocycler, using 2 mL of the overnight culture was used to inoculate 200 mL of LB and incubated with agitation for 16 hours at 37°C. Positive clones that harbor the gene in the sense orientation with respect to the T7 promoter were selected by enzymatic digestion, creating pET-Blue1/Lcyb1. Functional assays were carried out in Escherichia coli BL21 gold strain transformed with the pDS1B or pDS1BAet1 plasmids. pDS1B carries the carotenogenic genes of Enterina uredovora required to produce β-carotene [45]. The pDS1BAet1 vector has a mutation in the crtI (Lcyb) gene, which leads to the accumulation of lycopene in this strain [43]. This mutant strain was transformed with pET-Blue1/Lcyb1 and the empty, pET-Blue1 vector as a control. The transformed colonies were selected in LB medium supplemented with ampicillin (100 μg mL⁻¹) and chloramphenicol (50 μg mL⁻¹), after an incubation of 96 hours at 30°C. An overnight liquid culture of the mutant strain transformed with pET-Blue1/DeLcyb1or pET-Blue1 plasmids was used to inoculate 5 mL of LB medium supplemented with the aforementioned antibiotics and incubated with agitation for 16 hours at 37°C. Subsequently, 2 mL of the overnight culture was used to inoculate 200 mL of LB medium with the selective antibiotics. When the culture reached OD600: 0.6, 100 μL of 1 M IPTG (isopropyl β-D-thiogalactoside) was added to induce expression of the gene. The optical density of the culture was measured at OD600.

Table 1. Gene-specific primers used for functional characterization of DcLcyb1.

| Gene    | Primer name | Sequence (5′-3′) | Purpose |
|---------|-------------|-----------------|---------|
| Lcyb    | Lcyb1Fq     | tgaagctgcttacacctactgatta | To measure the expression of DcLcyb1 gene by qRT |
|         | Lcyb1Rq     | aactgcaagaaatgtaaggctaga |         |
| Psy1    | Psy1Fq      | atgctgatgagcattttaccctttc | To measure the expression of DcPsy1 gene by qRT |
|         | Psy1Rq      | ttctaatgctgtgcttccaaggggcttg |         |
| Psy2    | Psy2Fq      | gctaaacttcctcggttgcgtctggcttgta | To measure the expression of DcPsy2 gene by qRT |
|         | Psy2Rq      | gctcagatgatgguagtgtct |         |
| Lcyb1   | Lcyb2Fq     | ggattctctgtgctttacacttccgcag | To measure the expression of DcLcyb2 gene by qRT |
|         | Lcyb2Rq     | cccagaaagacac |         |
| Ubi     | ubiF        | gctgagagccgagcaaac | Normalizer gene for qRT experiments |
|         | ubiR        | cttggctggttgtgagctc |         |
| Lcyb    | Lcyb1Fatg   | atgaaagctgcttacactttactc | To amplify the complete cDNA of DcLcyb1 for over expression and complementation |
|         | Lcyb1R     | cttcacaagacttttgaaag |         |
| Lcyb    | Lcyb5S      | gaaattatatgtggtggtggtatcga | To amplify a coding DcLcyb1 fragment for silencing |
|         | Lcyb5R      | gaaattgcaagtatgggtgatttaactg |         |
| Hpt1f   | Hpt1F       | ttggtgacagccgcaaggtgtgaa | To determine transgenic lines |
|         | Hpt1R       | aagactgctgctgctgtgcag |         |
| Lcyb    | Lcyb1i      | ggtgacagcagctgccacacac | To determine DcLcyb1 silenced lines |
|         | Lcyb1Ri     | gctcctgctgtactccttc |         |
| Lcyb    | LcybF       | Atgaaagctgcttacactttactc | To amplify DcLcyb1 gene for sub-cellular localization |
|         | LcybR       | cttcacaagacttttgaaag |         |
was added to half of the culture to induce the expression of the gene and the other half was used as control. All the assays were performed in triplicate and in darkness, in order to maximize carotenoid production.

Vector construction for post-transcriptional gene silencing and over-expression of DcLcyb1

For post-transcriptional gene silencing, a 483 bp fragment of DcLcyb1 cDNA (798 bp-1280 bp) was amplified from carrot leaf RNA with LCYBPS and LCYBRs (Table 1) and cloned in the EcoRI site of pUCpSS. Positive clones were analyzed by PCR and enzymatic digestion. After sequencing, clones with the DcLcyb1 fragment in an antisense (AS) and in a sense (S) orientation with respect to the double 35ScaMV promoter (d35S) were digested with HindIII. The resulting d35S::LcybAS (antisense) and d35S::LcybS (sense) fragments were purified and cloned in the HindIII restriction site of the binary vector pBIN19, forming pBLcybAS and pBLcybS. For DcLcyb1 over-expression, the complete DcLcyb1 coding sequence (DQ192190) cloned into pCR8/III restriction site of the binary vector pBIN19, forming pBLcybAS and pBLcybS. For DcLcyb1 over-expression, the complete DcLcyb1 coding sequence (DQ192190) cloned into pCR8/GW/TOPO (Invitrogen) as described before (pCR8/Lcyb), was recombined into the binary vector pMDC32 [46], following the manufacturer’s instructions to produce pMDC32/DcLcyb1 in which DcLcyb1 is inserted under the control of a d35ScaMV promoter.

Agrobacterium tumefaciens-mediated transformation of Daucus carota

The binary vectors pBLcybAS, pBLcybS and pMDC32/DcLcyb1 were transformed into Agrobacterium tumefaciens (strain GV3101). D. carota transformation was achieved following the protocol described by Chen and Punja, 2002 [47]. Briefly, hypocotyl segments of 14 day-old seedlings were co-cultivated with Agrobacterium carrying the vector of interest, and placed on solidified MS media containing 1 mg/L 2.4D for somatic embryogenesis induction and (4.4 g/L MS salts, 20 g/L sucrose and 0.7% agar) in darkness. After 2 days, the explants were transferred to solid MS medium containing 1 mg/L 2.4D for somatic embryogenesis induction and supplemented with 50 mg/L kanamycin (for pBLcybAS and pBLcybS) or 50 mg/L hygromycin (for pMDC32/DcLcyb1) and 200 mg/L carbenicillin. After four weeks in darkness, the explants were placed on solidified MS medium containing 0.5 mg/L 2.4D, 100 mg/L kanamycin (for pBLcybAS and pBLcybS) or 100 mg/L hygromycin (for pMDC32/DcLcyb1) and 200 mg/L carbenicillin in photoperiod conditions (16 hrs light, 115umol/m2/sec). Antibiotic-resistant embryos were transferred to MS media in the absence of hormones to induce the development of shoots. After six months, transformed plantlets were transferred to soil in a temperature and photoperiod controlled greenhouse (16 hrs light, 115 umol/m2/sec). PCR amplification of hptII was carried out to select transgenic lines. DcLcyb1qRT was performed using LybFq and Lycb/Rq primers to select over expresser transgenic plants. Lines with reduced levels in DcLcyb1 expression were obtained through conventional RT-PCR using previously described hptII primers and FLcyb (5’-tgatctggatgatctcag-3’) and RLcyb (5’-gtgctgatcggcttc-3’) primers, which amplify a 760 bp fragment that contains the 483 bp fragment used as target to induce silencing of the gene.

DcLcyb1 sub-cellular localization

The full length DcLcyb1 coding sequence was amplified without the stop codon from a sequenced pCR8/DcLcyb1 clone using Elongase enzyme and LybF and LCFR primers (Table 1) and subcloned into pCR8 (Invitrogen). Positive clones were sequenced in Macrogen Corp. Gateway technology was used to recombine pCR8/Lcyb1 into the binary vector pMDC85 [46] to obtain the chimeric protein Lcyb1-GFP directed by ad35ScaMV promoter. The rec:A::YFP (kindly provided by Dr. Lee Meisel) harbors the chloroplastic destination peptide in frame with the YFP gene [48]. The constructs were transiently expressed in leaves of 2 month-old N. tabacum plants by agrofiltration according to [49]. The samples were visualized in an inverted Epifluorescence Microscope (IX-70, Olympus America Inc., Melville, NY). The GFP signal was measured at Ex 480/30X, Dichroic 505DCLP, Em 535/40nm and processed with LSM5 Image Browser and Adobe Photoshop software.

Carotenoid extraction and High Performance Liquid Chromatography (HPLC)

Carotenoids from wild-type and transgenic carrots were extracted from 100 mg of leaves or roots with 1 ml of hexane/acetone/ethanol (2:1:1 v/v) as described in [41]. Two successive extractions were performed to remove carotenoids until the tissue was blanched. The extract was dried with N2 and resuspended in 1 ml of acetone. Carotenoids from E. coli BL21 complemented cells were extracted from 70 mL of liquid cultures centrifuged at 5000 rpm for 15 minutes. The bacterial pellet was washed twice with cold sterile water and resuspended in 1 mL of sterile water with 500 µL of glass pearls. After vigorous mixing, 1 mL of acetone was added. Afterwards, the solution was again mixed vigorously and centrifuged at 4000 rpm for 5 minutes. The collected aqueous phase was washed twice with acetone and mixed vigorously with 4 volumes of petroleum ether. This solution was centrifuged and the upper phase collected in glass tubes, dried with gaseous nitrogen, resuspended in 100 µL of acetone and kept at −80°C until total carotenoid and carotenoid composition analysis was performed. Total carotenoids from plants and bacteria were measured by spectrophotometry at 474 nm in Shimadzu HPLC equipment (LC-10AT) with a diode array and the data analysis was carried out with the LCsolution® software program. For phytene measurements, chromatograms at 285 nm were obtained. These pigments were separated by a HPLC using a RP-18 Lichrocart125-4 reverse phase column (Merck®, utilizing an acetonitrile:methanol:isopropanol (85:10:5 v/v) mix as a mobile phase with a 1 ml/min flow rate at room temperature in isocratic conditions. The elution spectra of each maximum were obtained using a diode array detector. The carotenoids were identified according to their absorption spectra, retention time and comparison with specific pigment standards, which was corroborated by comparison with the Carotenoids Handbook [50,51]. All operations were carried out in triplicate, on ice and dark conditions to avoid photodegradation, isomerization and structural changes of carotenoids.

Results

In silico analysis and sub-cellular localization of DcLcyb1

The sequence analysis shown in Figure 2A indicates that DcLcyb1 has conserved motifs related to lycopene β-cyclases such as a cyclase motif I and II (CM1 and CMII), a LCY specific motif, a conserved region β-LCY, a di-nucleotide binding site, a charged region and the β-LCY motif, β-LCY CAD region (Catalytic Activity Domain), domains described as essential for lycopene β-cyclase activity [17,22,23,32].

DcLcyb1 has high amino acid identity with β-lycopene cyclase of tomato (SILCYB1, 82.4%, [28] and pepper (CaLCYB1, 80.7%, [22]. The most variable region of DcLcyb1 is found at the N-terminus (Figure 2A) in which a plastid targeting sequence of 68 amino acids is predicted (ChloroPv1.1®).
Functional Characterization of Carrot LCYB1

**A**

Conserved Region β-LCY's

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 71        | 71        | 71        |

Di-nucleotide binding site

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 78        | 78        | 78        |

LCY's specific motif

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 117       | 117       | 117       |

CMI

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 127       | 127       | 127       |

β-LCY CAD

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 288       | 288       | 288       |

CMI

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 289       | 289       | 289       |

β-LCY CAD

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 309       | 309       | 309       |

CMII

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 351       | 351       | 351       |

β-LCY CAD

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 410       | 410       | 410       |

Charged region

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 408       | 408       | 408       |

β-LCY's motif

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 468       | 468       | 468       |

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 500       | 500       | 500       |

| DcLCYB1   | S1LCYB1   | CaLCYB1   |
|-----------|-----------|-----------|
| EFSGQFV   | EFSGQFV   | EFSGQFV   |
| 486       | 486       | 486       |

**B**

**a, d** Micrographs of the leaf with green fluorescence indicating the presence of β-carotene.

**b** Magnified view showing the distribution of green fluorescence spots.

**c** Detailed view highlighting specific green fluorescence spots.

**e** Top-down view with arrows pointing to areas of interest.

**f** Close-up view with arrows indicating specific areas.
To confirm these in silico findings, a DcLCYB1::GFP fusion protein was constructed under the control of a d35SCaMV promoter in order to determine the sub-cellular localization of the enzyme experimentally. The DcLCYB1::GFP fusion protein was transiently expressed in leaves of 2 month-old tobacco. Epifluorescence laser microscopy of leaves post-infiltration showed that transiently expressed in leaves of 2 month-old tobacco. Epifluorescence laser microscopy of leaves post-infiltration showed that the known chloroplast-targeted protein, RECA fused to YFP (Figure 2B; [48]).

**Functional analysis of DcLcyb1 by means of heterologous complementation**

In order to evaluate the function of DcLcyb1, an in vivo analysis through heterologous complementation of the pDS1B ΔactY E. coli BL21 gold strain [45] was performed. This mutant strain carries a plasmid (pDS1B) harboring the genes from *Eucnica umedowana* necessary for β-carotene synthesis, and a mutation in *Lcyb* (citI) gene leads to the accumulation of lycopene. The strain was co-transformed with pET-Blue1/DcLcyb1 and as a negative control with pET-Blue1. Carotenoids were extracted from liquid bacterial cultures (48 hours culture), and analyzed by reverse phase HPLC. The chromatogram obtained from mutant strains co-transformed with pET-Blue1/Lcyb1 in the presence of IPTG showed a peak with a characteristic absorbance spectrum corresponding to β-carotene that is absent in the control strain (Figure 4). The quantification of the HPLC chromatogram showed that the complemented strain (pDS1B ΔactY/pET-Blue1/DcLcyb1) presents significantly higher levels of β-carotene accompanied by a diminished level of lycopene compared to the control strain pDS1B ΔactY/pET-Blue1 (not shown). Our findings show that DcLcyb1 is able to restore the carotenoid biosynthetic pathway in the pDS1B ΔactY E. coli strain, leading to β-carotene production.

**Over-expression of DcLcyb1 in D. carota increases carotenoid levels and affects endogenous carotenogenic gene expression**

To evaluate the function of DcLcyb1 in planta, the ORF was cloned in the binary vector pMDC32 and transferred by Agrobacterium-mediated transformation into *D. carota*. The transformed lines were verified by PCR amplifying *hptII* gene, and three independent transgenic lines were selected and subjected to qRT, to evaluate DcLcyb1 expression (Figure 5A and B). In leaves, the expression of DcLcyb1 was 2 to 8 fold greater than that observed in wild type plants (Figure 5A), and in roots of the transgenic lines, an increment of 1.1 to 2.3 fold in the expression of DcLcyb1 related to the roots of wild type plants was obtained (Figure 5B). On analyzing carotenoid pigments, the over expression of this gene induced increments of 1.6 to 1.8 fold in total carotenoids and 2.6 to 2.8 fold in β-carotene levels in leaves, compared to wild-type plants (Figure 5C). A modification of the other branch of carotenoid biosynthesis was observed in leaves of some lines, where α-carotene levels were reduced until -5 fold (Figure 5C), reflecting a possible function of DcLcyb1 in both branches of the carotenogenic pathway, redirecting the flux to the synthesis to β-carotene. In the storage root, the total carotenoid and β-carotene levels in over expresser lines increased 1.1 to 1.8 fold, and 1.2 to 2 fold, respectively, with respect to the wild-type plants, while α-carotene levels changed between -0.5 to 1.5 fold (Figure 5C and D).

As expected, chlorophyll content was also increased in the transgenic lines (Figure 6) because chlorophyll and carotenoid...
synthesis is coordinately regulated. Phytoene levels did not change in transgenic lines related to wild type plants (not shown), despite the increment in expression levels of DcPsy1 and DcPsy2 (Figure 5E and F). This could be associated with the increment in DcLcyb1 and DcLcyb2 expression that may enhance the flux to β-carotene synthesis. The phenotype of whole and cross-sectioned transgenic storage roots, shown in Figure S1, showed that they differed in thickness and color with respect to the wild-type carrots, and correlated with the carotenoid content, especially in L6 (Figure 5D). To analyze the effect of DcLcyb1 over-expression in the carotenogenic pathway, the transcript levels of endogenous carotenogenic genes were evaluated. DcPsy1, DcPsy2 and DcLcyb2 genes were significantly induced in leaves and storage roots of the three transgenic lines (Figure 5E and F). In leaves, induction of DcPsy1 was greater than that of the paralog gene, DcPsy2 (5.8–8.4 fold versus 1.2–2.5 fold, respectively). In roots, L6 and L8 transgenic lines, which presented the greatest increase in carotenoid levels in carrot roots, the DcPsy2 and DcLcyb2 genes were induced 3.5 to 4 fold related to wild-type storage roots, whereas DcPsy1 was induced by only 1.2 to 2.5 fold. These results show that the over expression of DcLcyb1 in carrot produces metabolic changes and transcriptional modulation of the carotenogenic pathway, which in turn leads to an increment in total carotenoids and β-carotene.

Post-transcriptional gene silencing of DcLcyb1 affects carotenoid levels and endogenous carotenoid gene expression

A post transcriptional gene silencing strategy was used to confirm the previous results, and to evaluate whether this gene has an organ specific function in carrot. Six silenced lines were obtained through qRT and three of them were analyzed in detail. The AS23, AS37 and AS48 silenced lines showed between 80% and 95% reduced expression of DcLcyb1 in leaves and roots (Figure 7A and B). In lines with reduced expression of DcLcyb1, the carotenoid levels were reduced by 70% in leaves and by 55% in the storage root, and β-carotene levels were reduced by 77% and 58% in leaves and roots, respectively (Figure 7C and E). In addition to carotenoid reduction, a pronounced reduction in storage root thickness and color was obtained in transgenic silenced lines respect to wild type carrots (Figure S1) in which the narrow root reaches half of wild type thickness. As changes in root phenotype and in carotenoid levels in leaves and storage roots of the silencing lines were observed, these results suggest that DcLcyb1 is involved in carotenoid biosynthesis in the entire carrot plant. The carotenoids from the other branch, especially lutein, were also reduced in leaves of the silenced plants (Figure 7C). These results suggest that the endogenous carrot DcLcyb1 is required for the correct synthesis of carotenoids of both branches (β-carotene and α-carotene) in leaves. However, in roots, the reduction in DcLcyb1 expression did not significantly affect the amount of α-carotene in silenced lines (Figure 7D), but the phytoene level was reduced significantly (Figure 7E) in a direct correlation with the endogenous gene expression of DcPsy1 and DcPsy2 (Figure 8). A
A 30% to 40% decrease in chlorophyll levels was obtained in leaves of carrots with reduced DcLcyb1 levels (Figure 6), which correlates with the results obtained for the over expresser DcLcyb1 lines. In these analyses, the S5 line transformed with the sense construct (pBLcyb8) was used as silencing control and exhibited a very similar expression level of DcLcyb1 compared to the wild-type, and consequently, the production of total carotenoids and β-carotene were not significantly different.

Figure 5. Over expression of DcLcyb1 increases carotenoid and β-carotene levels and expression of key carotenogenic genes.
Relative expression of DcLcyb1 gene in L6, L8 and L9 from (A) leaves and (B) storage roots of transgenic carrot plants. Ubiquitin was used as normalizer in qRT measurements. Carotenoids of D. carota L6, L8 and L9 transgenic lines were extracted from (C) leaves and (D) storage roots and carotenoid composition was determined by spectrophotometry and High Performance Liquid Chromatography. Relative expression of DcPsy1, DcPsy2 and DcLcyb2 in transgenic carrot lines from (E) leaves and (F) storage roots. Ubiquitin was used as normalizer in qRT measurements. For gene expression and carotenoid analysis three months-old plants were used. Columns and bars represent the means and SE (n = 3). Asterisks indicate significant differences between transgenic lines and the wild type plant. Non-paired one and two tailed t-tests (p < 0.05) were performed for all the transgenic lines and the wild type plant.

doi:10.1371/journal.pone.0058144.g005
The effect of DcLcyb1 silencing on the expression of other genes involved in the carotenogenic pathway was analyzed in the storage roots of transgenic lines (Figure 8). In storage roots, the transgenic lines presented a general decrease in carotenogenic transcript levels, especially in DcPsy2 and DcLcyb2 in AS37 and AS48 where a reduction of 98% (48 fold) to 69% (3.2 fold) was observed for DcPsy2 and between 98% (48 fold) and 83% (6 fold) for DcLcyb2. This result correlated with the lower carotenoid and phytoene accumulation in these silenced carrot roots (Figure 7D and E).

Therefore, the reduction of DcLcyb1 expression in carrot led to consistent effects over the expression of key carotenogenic genes which could be in part responsible for the alterations in the carotenoid accumulation observed in those lines.

Discussion

The enzymatic reaction that gives rise to \( \alpha \)-and \( \beta \)-carotene pigments is a key regulatory branching point performed by the lycopene \( \beta \)-and \( \alpha \)-cyclases (LCYB and LCYE) in plant plastids [10,20,38,35,56]. LCYB is encoded by single genes in several plants, including Arabidopsis, maize and rice [17,24,26,57], or by small gene families in others, like tomato, pepper, papaya and orange [16,28,31,33,42,52,58]. In most of these plants, organ specific functions of LCYB genes have been described.

In carrot, \( \beta \)-carotene and \( \alpha \)-carotene are the major carotenoid components in a mature storage root, whose levels increase significantly during root development reaching 600 \( \mu \)g/g dry weight (dw) and 300 \( \mu \)g/g dw, respectively [43,59]. This increase indicates an important stimulation of the \( \beta \)-\( \beta \)-cyclization of lycopene in the carrot carotenogenic pathway during storage root development. In carrot, two Lycb genes have been identified, named DcLcyb1 and DcLcyb2. Quantitative analysis indicated that Lcyb1 expression is induced in storage roots during carrot development whilst Lcyb2 is not [41]. The expression of DcLcyb1 correlates with the accumulation of carotenoids in mature carrot roots [41]. DcLcyb1 is also induced in leaves during carrot development, and reaches a higher level of induction in these organs than in the storage root in a mature plant (Figure 3), although the amount of carotenoids in leaves remains constant [41]. This could be associated with the photo-protective function in which carotenoids are involved in leaves. Carotenoids protect cells from the effects of excessive light exposure, which rises the carotenoid turn over in leaves which in turn could affect the expression level of the corresponding genes.

In roots, DcLcyb1 expression increases 14-fold during development, and is one of the most highly-induced carotenogenic genes (Figure 3, [41]) suggesting that DcLcyb1 is an important control point in this pathway. This gene expression profile correlates with that in the orange stage of Satsuma mandarin and Valencia orange fruit ripening, where a simultaneous increase in the expression of CitPsy, CitPsy, CitHyb, and CitZp genes led to a high accumulation of xanthophylls in the flavedos and juice sacs of the citrus fruits [60].

Here, we functionally characterized the lycopene \( \beta \)-cyclase 1 gene of D. carota in order to determine whether this gene is involved in the biosynthesis of carotenoids in carrot. First, we analyzed whether DcLCYB1 possesses the conserved regions characteristic of plant LCYBs, by comparing the amino acidic sequence of DcLCYB1 with the chloroplast specific SILCYB (Solanum lycopersicum LCYB) and CaLCYB (Capsicum annum LCYB) which belong to plants that present high carotenoid accumulation. The in silico analysis of DcLCYB1 showed that it harbors the conserved motifs of the other plant LCYBs (Figure 2A). For example, the three \( \beta \)-LCY CAD regions that were reported as essential for lycopene \( \beta \)-cyclase catalytic activity are fully conserved in DcLCYB1 [14,22,23]. In the “conserved region \( \beta \)-LCYs”, which has been proposed as essential for the association of LCYB to membrane components and also for its catalytic activity [14,22], there are four amino acid changes in carrot LCYB1 compared to the sequences of SILCYB and CaLCYB. Specifically, these are a glutamic acid instead of a proline at position 60, a glutamine instead of asparagine at position 68, an asparagine instead of a glutamine at position 70 and a leucine instead of metionine at position 73 (Figure 2A). In addition, the “\( \beta \)-LCYs motif” also possesses a single amino acid change that affects the amino acid charge at position 446, where an aspartate conserved in pepper and tomato sequences is replaced by a serine in the DcLCYB1 sequence from carrot (Figure 2A). However, the possible amino acidic changes identified in silico, did not alter the functionality of DcLCYB1, as shown by in vivo experiments carried out in the present article. The specific impact of the different amino acid substitutions identified in silico on lycopene \( \beta \)-cyclase catalytic activity requires further characterization using site directed mutagenesis.

Bioinformatic analysis using ChloroPv1.1 showed that the DcLCYB1 protein sequence presents the signal peptide in its N-terminal, as found in other plant carotenogenic enzymes [8,19,21]. In agreement with the in silico analysis, the sub-cellular localization carried out by means of a chimeric LCYB1-GFP fusion protein in tobacco leaves, confirmed the chloroplast localization of this enzyme (Figure 2B). Carotenoid enzymes are found in plastids (especially in chloroplasts and chromoplasts) of plant cells [39], where they exert their function. This result supports the proper organelle localization for this enzyme and suggests that DcLCYB1 functions correctly, which was directly proven by means of the heterologous complementation system in E. coli. This methodology has been reported previously to be useful in determining carotenogenic protein functionality [31,52,61]. As expected, the DcLCYB1 protein was able to restore the normal course of the carotenogenic pathway leading to the production of \( \beta \)-carotene using lycopene as substrate in E. coli (Figure 4). In addition to validating the utility of this heterologous system for determining the activity of a plant carotenogenic enzyme, these results proved that the DcLcyb1 encodes for an enzyme with lycopene \( \beta \)-cyclase activity.

Figure 6. Chlorophyll content in over expresser and silenced DcLcyb1 lines of carrot plants. Chlorophyll amount in leaves of over expresser and silenced lines was determined by spectrophotometry and HPLC. The chlorophyll amount in wild-type was set at 100%. For this analysis, leaves of three month-old plants were used. Columns and bars represent the means and SE (n = 3). Asterisks indicate significant differences between transgenic lines and the wild type plant. No paired one and two tailed t-tests (p < 0.05) were performed for all the transgenic lines and the wild type plant.

doi:10.1371/journal.pone.0058144.g006
In order to determine the functionality of this gene in plants, we used two different approaches, over-expression and post-transcriptional gene silencing of \textit{DcLcyb1} in carrot, since these techniques have been used elsewhere to evaluate gene function \textit{in planta} [28,33,40,62]. The stable over-expression of \textit{DcLcyb1} in carrot led to an increase in total carotenoids and \(\beta\)-carotene levels in leaves and carrot roots, respectively (Figure 5C and D), which is correlated with \textit{DcLcyb1} transcript levels in leaves and roots Figure 7. \textit{DcLcyb1} silenced lines have diminished carotenoid and \(\beta\)-carotene levels in leaves and roots. \textit{DcLcyb1} transcript levels in (A) leaves and (B) storage roots in carrot transgenic lines were evaluated by qRT. \textit{Ubiquitin} was used as normalizer. Carotenoid quantification in (C) leaves and in (D) storage roots of wild-type (WT) and silenced lines was determined by spectrophotometry and HPLC at 474 nm. (E) Phytoene in storage roots of wild-type (WT) and silenced lines was quantified by HPLC at 285 nm. For gene expression and carotenoid analysis, three months-old plants were used. Columns and bars represent the means and SE (n = 3). Asterisks indicate significant differences between transgenic lines and the wild type plant. Non-paired one and two tailed t-test (p < 0.05) were performed for all the transgenic lines and the wild type plant. doi:10.1371/journal.pone.0058144.g007

Figure 7. \textit{DcLcyb1} silenced lines have diminished carotenoid and \(\beta\)-carotene levels in leaves and roots. \textit{DcLcyb1} transcript levels in (A) leaves and (B) storage roots in carrot transgenic lines were evaluated by qRT. \textit{Ubiquitin} was used as normalizer. Carotenoid quantification in (C) leaves and in (D) storage roots of wild-type (WT) and silenced lines was determined by spectrophotometry and HPLC at 474 nm. (E) Phytoene in storage roots of wild-type (WT) and silenced lines was quantified by HPLC at 285 nm. For gene expression and carotenoid analysis, three months-old plants were used. Columns and bars represent the means and SE (n = 3). Asterisks indicate significant differences between transgenic lines and the wild type plant. Non-paired one and two tailed t-test (p < 0.05) were performed for all the transgenic lines and the wild type plant. doi:10.1371/journal.pone.0058144.g007
(Figure 5A and B). The proportion of β-carotene and α-carotene is mostly determined by the comparative amounts and/or activities of the LCYB and LCYE enzymes [26,28,63,64,65]. Transgenic carrots that overexpress DeLcyb1, redirect the carotenoid flux to β,β-carotene, diminishing the amount of β,α-carotene molecules.

In wild type and transgenic carrots, the carotenoid levels in roots are higher than in leaves although the expression of DeLcyb1 is lower in this organ than in leaves (Figures 5, 5A and B). This was also observed for other carotenogenic genes [41] because total carotenoid levels, including β,α-carotene accumulation, are determined by the total flux in the pathway in which enzymatic activity and stability may also exert an important role. In addition, in leaves, the carotenoid levels are the net result of the synthesis and degradation of carotenoids exerted by light through photo-oxidative processes [66], whereas in roots the carotenoids are stored in stable structures (plastoglobuli and crystals), present in chromoplasts that could diminish the carotenoid degradation rate [40].

In addition, when DeLcyb1 was over-expressed in carrot, a side effect on the transcript levels of endogenous carotenogenic genes was observed (Figure 5E and F). In carrot leaves and roots, the DePsy1, DePsy2 and DeLcyb2 genes were also induced in transgenic lines. Romer et al., 2000 [67] also obtained an induction in endogenous Splds, SlZds and SlLcyb expression on overexpression of 35S:crtI in tomato. In contrast, the over expression of AtLcyb in tomato under a Pd6 promoter, did not affect significantly the expression of endogenous carotenogenic genes, although the fruits contained more lycopene and β-carotene [68]. Thus, a direct correlation between the increase of DeLcyb1 transcript levels (Figure 5A and B), the total carotenoid and β-carotene levels (Figure 5C and D), and the up regulation in the expression levels of DePsy1, DePsy2 and DeLcyb2 (Figure 5E and F) is observed.

When a bacterial crtB gene under a root specific promoter was expressed in the white QAL carrot variety, an increment in crystal accumulated-carotenoids was obtained [40]. However, these carrot lines were enriched in carotenoid intermediates such as phytolene, phytofluene and ζ-carotene [40]. In DeLcyb1 over expresser lines, phytolene was not altered, but the levels of the direct products of Lcyb, which include ζ-carotene and β-carotene, were significantly increased. This result suggests that DeLcyb1 might act as a carotenogenic pathway regulator, possibly through an indirect positive feedback mechanism over the expression of other carotenogenic gene(s), which further increases carotenoid accumulation in transgenic carrot plants.

In over-expressing lines L6 and L8, which displayed the highest carotenoid and β-carotene level, the expression of DePsy2, and not DePsy1, was significantly induced in the storage root, which could also assist in raising the carotenoid level in this organ. Moreover, DePsy1 is induced in leaves of these transgenic lines, which suggests that the requirement of both organs regarding DePsy induction through DeLcyb1 should be different. Recently, we determined that DePsy2 is preferentially expressed during carrot root development, while DePsy1 is mostly highly expressed in mature leaves [41], findings which correlate with the results presented here.

On the other hand, post-transcriptional gene silencing of DeLcyb1 showed that this gene is necessary for β-carotene synthesis in leaves and in roots of D. carota (Figure 7C and D), and especially in leaves, correlating with the highest endogenous expression level (Figure 3). The two transgenic lines (AS37, AS48), which presented the most substantial decrease in DeLcyb1 expression levels in leaves and roots (more than 90% of gene silencing), showed up to a 77% reduction in β-carotene in photosynthetic organs and up to a 58% decrease in the storage root (Figure 7C and D). In addition, we observed a significant reduction in lutein levels in leaves, although no differences were observed in the level of α-carotene in these organs (Figure 7C). This could be explained because the activity of other enzymes, such as DeLCYE and/or DeCHBX, might not be being affected by the reduction of DeLcyb1 expression. Interestingly, phytoene levels decreased between 2 and 3 fold in roots of the transgenic lines with reduced levels in DeLcyb1, consistent with a reduction in the expression of DePsy1 and DePsy2.

As mentioned above, carotenoids carry out a fundamental role during photosynthesis, being located in the photosynthetic membrane in carotenoid-chlorophyll complexes [10]. In addition, chlorophylls and carotenoids, that share GGPP as a common precursor, are coordinately regulated during de-etiolation and by the redox status [69,70]. Light is a stimulus that induces the expression of some carotenogenic genes and simultaneously activates the expression of Ppox (protoporphyrinogen IX oxidase), a gene that encodes a light- harvesting chlorophyll a/b binding protein [54,69]. The phytochrome system is involved in the control of carotenogenic gene expression, as a greater induction of
Psy and Lcyb transcripts was detected after exposure to red-light than to blue-light [69]. In addition, changes in carotenoid composition or in enzymes itself, alters plastid development [71]. Our results indicate that an increment or reduction in carotenoids, affects directly and significantly the amount of chlorophyll in transgenic plants, suggesting that an increase or reduction in DeLcyb1 expression, may alter the amount of a common precursor of both pathways. With these results, we suggest that DeLcyb1 is required for carotenoid accumulation in the whole carrot plant, and that it does not have an organ-specific function in this specie, unlike that reported previously for tomato SlLcyb and SlLcyb [28,58,64].

Moreover, the silencing of DeLcyb1 caused a significant decrease in the expression of endogenous carotenogenic genes in carrot roots, particularly in DePsy2 and DeLcyb2; correlating with the level of reduction in DeLcyb1 expression in the transformed carrot lines (Figure 8). The 483bp fragment used to induce DeLcyb1 silencing has just 58% nucleotide identity with DeLcyb2. Therefore, the RNAi strategy employed in this work would not have an effect on the expression of DeLcyb2 directly, and the reduction of DeLcyb2 expression determined by qRT was a consequence of DeLcyb1 silencing.

In conjunction with the alterations observed in the transgenic carrot plants which over-expressed DeLcyb1, our findings lead us to conclude that an increase or decrease in DeLcyb1 transcript levels produces not only changes in carotenoid accumulation but also in the thickness of storage root and expression of key endogenous carotenogenic genes. Taken together, these results demonstrate that DeLcyb1 encodes for a LCYB enzyme that is functional and a key player in the carotenogenetic pathway in *Daucus carota*. Further studies could be focused on the identification and characterization of the DeLcyb1 promoter to study the regulation of this gene during carrot development, in leaves and in the storage root.

**Supporting Information**

Figure S1 Storage root phenotype of DeLcyb1 over expresser and silenced lines. Pictures were taken in 3 months old representative carrots that were cultivated in a mix of soil and vermiculite (2:1) in a growth chamber with cool-white fluorescent light (115 µmol m⁻² s⁻¹). Horizontal bar: 2 cm. Vertical bar: 5 cm. (TIF)

**Acknowledgments**

We thank Daniel Laporte for critically reading the manuscript and Lee Meisel for providing the RECA::YFP construct.

**Author Contributions**

Conceived and designed the experiments: JCM CS. Performed the experiments: JCM LP PF. Analyzed the data: JCM LP PF CS. Contributed reagents/materials/analysis tools: VC MH CS. Wrote the paper: JCM CS.

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