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ORIGINAL ARTICLE

Novel *9-cis/all-trans* β-carotene isomerases from plastidic oil bodies in *Dunaliella bardawil* catalyze the conversion of *all-trans* to *9-cis* β-carotene

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Abstract

Key message **We identified and demonstrated the function of *9-cis/all-trans* β-carotene isomerases in plastidic globules of *Dunaliella bardawil*, the species accumulating the highest levels of 9-cis β-carotene that is essential for humans.**

Abstract The halotolerant alga *Dunaliella bardawil* is unique in that it accumulates under light stress high levels of β-carotene in plastidic lipid globules. The pigment is composed of two major isomers: *all-trans* β-carotene, the common natural form of this pigment, and *9-cis* β-carotene. The biosynthetic pathway of β-carotene is known, but it is not clear how the *9-cis* isomer is formed. We identified in plastidic lipid globules that were isolated from *D. bardawil* two proteins with high sequence homology to the D27 protein—a *9-cis/all-trans* β-carotene isomerase from rice (Alder et al. Science 335:1348–1351, 2012). The proteins are enriched in the oil globules by 6- to 17-fold compared to chloroplast proteins. The expression of the corresponding genes, *9-cis-βC-iso*1 and *9-cis-βC-iso*2, is enhanced under light stress. The synthetic proteins catalyze in vitro conversion of *all-trans* to *9-cis* β-carotene. Expression of the *9-cis-βC-iso1* or of *9-cis-βC-iso2* genes in an *E. coli* mutant line that harbors β-carotene biosynthesis genes enhanced the conversion of *all-trans* into *9-cis* β-carotene. These results suggest that *9-cis*-βC-ISO*1* and *9-cis*-βC-ISO*2* proteins are responsible for the formation of *9-cis* β-carotene in *D. bardawil* under stress conditions.

Keywords *9-Cis/all-trans* β-carotene isomerases · *Dunaliella bardawil* · Plastidic lipid globules

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Introduction

β-Carotene (βC) is an essential pro-vitamin for humans found primarily in fruits and vegetables. βC gives rise to *11-cis* retinal, our visual pigment, to retinoic acid (RA), an essential metabolite which exists in two isomeric forms and is involved in regulation of differentiation, the immune system and energy metabolism and also functions as an antioxidant providing protection against degenerative diseases including cardiovascular disease and cancer (Khoo et al. 2011; Eroglu and Harrison 2013). Epidemiological surveys and intensive research studies have shown that βC from the microalga Dunaliella differs from synthetic βC in its isomeric composition and in its biological effects. For example, synthetic βC may be a cancer promoter at high doses (Pastorino 1997), whereas natural βC is recognized as a cancer preventive and as protectant against cardiovascular, psoriasis, retinal diseases and other degenerative diseases (Harari et al. 2013; Rotenstreich et al. 2010, 2013; Zolberg Relevy et al. 2015). The reasons for these differences are believed to be the different isomeric composition of βC: whereas synthetic βC contains only the *all-trans* isomer, natural βC from some fruits and vegetables also contain small amounts of *cis* isomers, notable *9-cis* βC and *13-cis* βC. *D. bardawil* and *D. salina* teodoresco are the richest known natural sources for βC, amounting to 10% of their dry weight, and it consists of a 1:1 mixture of *all-trans*

and *9-cis* βC isomers (reviewed in Jin and Polle 2009). Humans need two βC isomers: *9-cis* and *all-trans. 9-cis* βC is a precursor of *9-cis* RA, whereas *all-trans* βC gives rise to *all-trans* RA. It has been well established that *9-cis* RA and *all-trans* RA interact with different receptors (retinoic acid receptor, RAR and the retinoid receptor, RXR, respectively), and control different essential processes in the body: *all-trans* RA is involved primarily in differentiation whereas *9-cis* controls primarily energy metabolism (Plutzky 2011).

The biosynthetic pathway of βC in *Dunaliella* is well established, and it consists of synthesis of isopentenyl pyrophosphate (IPP) through the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, condensation of the C_5 IPP into C_{40} phytoene followed by desaturation and cyclization which take place in the chloroplast (reviewed by Jin and Polle 2009). The mechanism of formation of *cis* βC isomers in plants and algae is not entirely clear. βC isomerization may occur spontaneously during food storage and processing (Tang and Chen 2000; Marx et al. 2003; Vasquez-Caicedo et al. 2007) or may be induced by heating, by light or by triplet-state sensitization (Kuki et al. 1991). Plant carotenoid biosynthesis pathway proceeds via *15-cis* carotenoid intermediates that serve as substrates of phytoene and ζ-carotene desaturases (Breitenbach and Sandmann 2005; Beltran et al. 2015). Previous studies have not clearly established how *9-cis* βC is formed in *Dunaliella*. The only relevant study, using different inhibitors of carotenoid biosynthesis, came to the conclusion that the *all-trans* to *9-cis* isomerization occurs at an early stage in the biosynthetic pathway, probably around phytoene (Shaish et al. 1990). This was based on identification of two isomers of the different intermediates, but it was not clearly established that the second isomers are indeed *9-cis*. Recently, Alder et al. identified a protein that catalyzes *all-trans*/*9-cis* βC isomerization (Alder et al. 2012) which has been originally identified as part of the strigolactone biosynthesis pathway in *Oryza sativa* and was termed D27 (Lin et al. 2009).

We have recently shown, by a proteome analysis of plastidic oil globules isolated from *D. bardawil*, that all the enzymes in the biosynthetic pathway from phytoene to βC are present in the plastidic oil globules, and that they are induced under light stress, indicating that the last stages of βC biosynthesis starting from phytoene are carried out within the lipid globules (Davidi et al. 2015). In addition to these βC biosynthetic enzymes, we also identified five putative βC isomerases. Two of these proteins showed a significant sequence homology to the D27. In this work we show that these proteins indeed catalyze *9-cis*/*all-trans* βC isomerization, indicating that they are responsible for the formation of *9-cis* βC in *D. bardawil*.

Materials and methods

Alga strain and growth conditions

Dunaliella bardawil is an isolated species (Ben-Amotz et al. 1989) deposited at the American Type Culture Collection (ATCC), Rockwille, MD, USA, No. 30861. Culturing conditions, growth media and N-limitation induction were as previously described (Davidi et al. 2014).

Preparation of lipid droplets and thylakoid membrane

Isolation of CLD and βC-plastoglobuli lipid droplets was performed essentially as previously described (Davidi et al. 2014).

Protein extraction for proteomic analysis

Proteins were extracted from isolated βC-plastoglobuli and the proteomic analysis, mass spectrometry analysis, data processing and searching by utilization on the proteome database that was constructed for *D. bardawil* were as previously described (Davidi et al. 2015). Proteins sequences were deposited in the *Dunaliella salina* proteome bank at the Weizmann Institute of Science as: *9-cis*-βC-ISO*1 9-cis*-βC-ISO2. These sequences are similar to the sequences Dusal.0248s00011.1 and Dusal.1143s00001.1 in *D. salina V1.0* in phytozome, respectively.

Dunaliella bardawil D27-like protein sequences were analyzed with BLASTP at the NCBI (Altschul et al. 1997) to find similar proteins in other species. The sequences were aligned with Clustal omega (Sievers et al. 2011) and D27 phylogenetic tree was generated using http://www.phylogeny.fr/ (Dereeper et al. 2008). Protein similarity networks was generated from an all-versus-all BLAST analysis (pairwise alignment between all pairs of proteins) of D27-like protein sequences and the network was created in Cytoscape version 2.8 (Smoot et al. 2011) with the BLAST2SimilarityGraph plug-in (Wittkop et al. 2011). Nodes (representing a protein) are connected with an edge (line) if the *E* value between two sequences was at least as good as the given value.

Gene expression of *9-cis-βC-iso1* and *9-cis-βC-iso2*

cDNA of *D. bardawil* grown in –N media was prepared as previously described (Davidi et al. 2012). *9-cis-βC-iso1* and *9-cis-βC-iso2* gene expressions were examined using *9-cis-βC-iso1* primers (F:5′- ATTTGAAGCCCCAGGAGACT, R:5′-CTGGGCAAAGAAGGTCTGAG) and *9-cis-βC-iso2* primers (F: 5′-GCAGACGCACAGTCAGTCAT and R: 5′-ACCGGCACTTTTTGATCTTG), and were compared with the expression of 18 S control gene.

Cloning of *9-cis/all-trans* βC isomerase genes

For plasmid construction full-length cDNA of *9-cis-βC-iso*1, *9-cis-βC-iso*2 were produced by RT-PCR using the following primer sequences: for *9-cis-βC-iso1* primers F: 5′-ATGCGGCAGGTTCGTCAGGG and R:5′-AAG CCTCAATCCCTTGGT were used; for *9-cis-βC-iso*2, F: 5′-ATGCTTGGTCGCTTGAGCTC and R: 5′-TTATGA TCGGCTCATCTTCGG. The cloned genes were inserted into pGEMT vector and cloned into *E. coli* cells. The plasmids were purified from the cells and sequenced to ensure correct sequence. Plasmid containing the *Oryza sativa* D27 gene (*OsD27* accession number FJ641055.1) was generated by Hy Laboratories Ltd (hylabs) in Rehovot, Israel, based on the sequence of Lin et al. (2009) with accession number FJ641055.1.

HPLC analysis

9-cis/all-trans βC resolution and spectral analysis was determined by reverse-phase HPLC (Waters e2695; Waters Corporation) using a 2.7-µm Halo C8 4.6×150-mm column (Advanced Materials Technology). The carotenoid extracts were dissolved in chloroform:methanol (1:1) and separated with isocratic methanol mobile phase at a flow rate of 0.8 mL/min at 25 °C. For peak detection, a photodiode-array detector (Waters Corp.) was used and peaks were identified by their absorbance spectra and from the absorbance quantified at 488 nm.

In vivo *9-cis/all-trans* βC isomerization assay

E. coli producing *all-trans*-βC was received from Prof. Joseph Hirschberg (Lotan and Hirschberg 1995) and their plasmids were extracted. For *in-vivo 9-cis/all-trans* βC-producing bacteria, plasmids of *9-cis-βC-iso1, 9-cis-βC-iso2* and *OsD27* genes were co-transformed with the *all-trans*-βC plasmids into *BL21 E. coli*. The βC production was induced by 0.5 mM IPTG for 24–48h. Then cells were pelleted and the carotenoid content was extracted from the cells by methanol–chloroform and the *9-cis/all-trans* βC ratio was determined by HPLC.

In vitro *9-cis/all-trans* βC isomerization assay

Plasmid construction, protein expression and the in vitro assay were performed essentially according to Alder et al. (2012). In brief, the recombinant proteins were generated by cloning *9-cis-βC-iso1, 9-cis-βC-iso2* and *OsD27* genes into pMAL-c4x (New England Biolabs) to obtain the maltose-binding (MBP) fusion proteins. Plasmids containing *9-cis-βC-iso1, 9-cis-βC-iso2* or *OsD27* genes fused to MBP were expressed in *E. coli* and MBP fusion proteins were purified using Amylose resin.

The enzymatic in vitro assay in brief: *all-trans* βC was incorporated by sonication into Triton X-100 micelles in 50% *n*-hexane and incubated in buffer containing 50 µM βC in 0.2% Triton X-100, 1 mM TCEP, 0.2 mM $FeSO_4$, 100 mM Na-HEPES, pH 7.8, 1 mg/ml catalase and 50 µM of MBP-purified proteins. The mixtures were incubated for 2h at 28 °C. Extractions were done in ethanol:*n*-hexane:water (1:1:1), the *n*-hexane phase was dried, dissolved in 100µL *n*-hexane and analyzed by HPLC.

Results

Identification and protein sequences of putative *9-cis* βC isomerases in *D. bardawil* oil globules

We have recently isolated and purified the β-carotene lipid globules from *D. bardawil* and made a comprehensive proteomic analysis of their protein content (Davidi et al. 2015). A major issue that came out of this work was the identification of phytoene desaturase (PDS), three isoforms of ζ-carotene desaturase (ζCDS) and lycopene cyclase (LCY), all the enzymes needed for conversion of phytoene into βC, within the plastidic oil globules. A second set of isoforms of the same proteins was identified in chloroplast membranes, suggesting that *D. bardawil* contains two biosynthetic pathways of βC biosynthesis in the chloroplast: a constitutive pathway in chloroplast membranes and an inductive pathway in lipid oil globules. In addition to these enzymes, we also identified 4–5 different proteins that show homology to carotene isomerases. Of particular interest were two different proteins that show significant sequence homology to *9-cis* βC isomerase from rice and *A. thaliana* termed D27 (Lin et al. 2009; Alder et al. 2012). Even though the overall amino acid sequence homology to *O. sativa* D27 protein is modest (31–33% sequence identity and over 50% sequence homology), the homology at the C-terminal domain, which is highly conserved in plants, is very significant (see underlined amino acids in Fig. 1). The enrichment factors of these proteins in lipid droplets in comparison to the total plastid proteome are 17.2 and 5.8 for *9-cis*-βC-ISO1 and *9-cis*-βC-ISO2, respectively, clearly suggesting that these enzymes localize in the βC lipid globules. We also identified a third D27-like protein that was termed *9-cis*-βC-ISO3, which shows significant sequence similarity to *9-cis*-βC-ISO2 (Fig. 2a, b). This protein, however, was not enriched in plastoglobules and, therefore, is not presented here. A search in the public *Dunaliella salina* database (Phytozome)

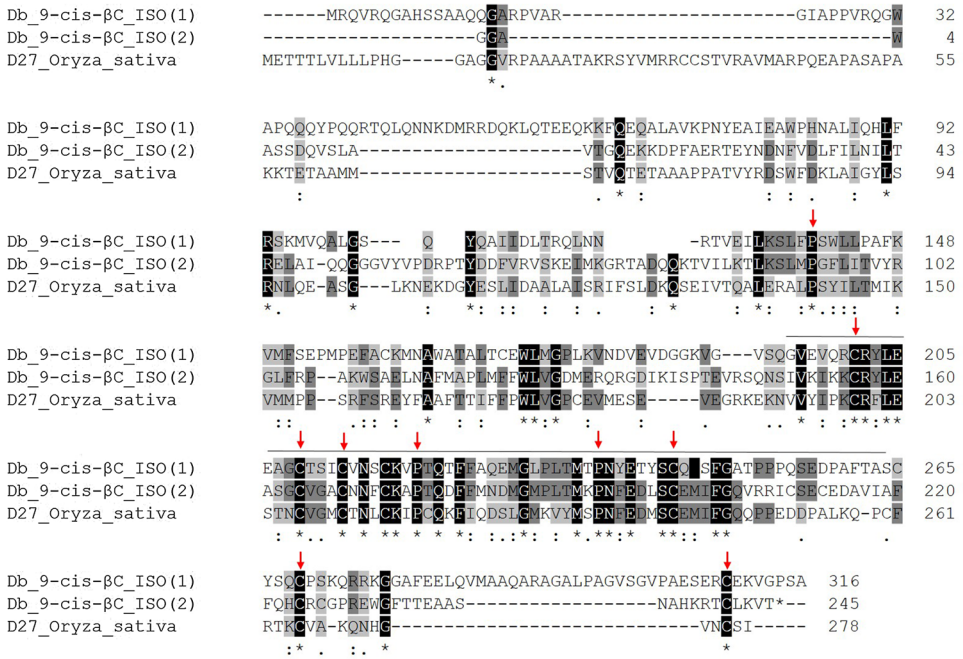

Fig. 1 Amino acid sequence alignments between *9-cis-βC-iso*1, *9-cis-βC-iso*2 and OsD27 from *Oriza sativa* (rice). The amino acid sequence alignments were made by CLUSTAL omega (1.2.0) program. Conserved cysteines (C) and prolines (P) were highlighted in *red*, to indicate structural conservation. Amino acid numbers 127–262 in *O. sativa* D27, which constitute the conserved domain in higher plants, were overlined. (Color figure online)

revealed the three D27-like proteins sequences that were marked Dusal.0248s00011.1, Dusal.1143s00001.1, Dusal.0074s00013.1 for *9-cis*-βC-ISO1, *9-cis*-βC-ISO2 and *9-cis*-βC-ISO3, respectively.

An extensive sequence homology search in different plant and algal genome, transcriptome and proteome database revealed that D27-like proteins are widely distributed from cyanobacteria to higher plants including green algae, red algae, diatoms and Cryptophyta (Fig. 2a, b). *9-cis*-βC-ISO2 and *9-cis*-βC-ISO3 resemble in sequence the majority of higher plants and algal D27 proteins whereas *9-cis*-βC-ISO1 seems to belong to a smaller sub-group of proteins, mostly from diatoms.

Induction of *9-cis-βC-iso*1 and of *9-cis-βC-iso*2 under light stress

In our previous study, we have shown that the expression of the βC biosynthesis enzymes in plastidic globules is activated during light stress and that the kinetics of activation proceeds in two stages, the first peaking around 12 h and the second around 96 h. Also the expression levels of mRNA of the two βC isomerases, *9-cis-βC-iso*1 and *9-cis-βC-iso*2, follows similar kinetics revealing two peaks at 12 h and at 96 h (Fig. 3). These results suggest that the expression of the two βC isomerases is synchronized with the upregulation of all other βC biosynthetic enzymes in the plastidic lipid globules and that they are part of the same biosynthetic pathway leading to formation of a mixture of two βC isomers, *all-trans* and *9-cis*.

Catalytic activity of *9-cis*-βC-ISO1 and *9-cis*-βC-ISO2

To test if *9-cis*-βC-ISO1 and *9-cis*-βC-ISO2 are indeed *9-cis*-βC isomerases, we took two approaches: the first was to express the genes in an engineered *E. coli* mutant that harbors all βC biosynthesis enzymes and produces *all-trans* βC and to test if the expression of these genes leads to conversion of part of the *all-trans* βC into *9-cis* βC. The second approach was to produce the synthetic proteins and to test in an in vitro assay if they catalyze conversion of *all-trans* βC into *9-cis* βC.

In the in vitro assay, synthetic proteins *9-cis*-βC-ISO1 and *9-cis*-βC-ISO2 were incubated with *all-trans* βC that has been pre-incorporated into Triton X-100 detergent micelles according to Alder et al. (2012). As shown in Fig. 4, 9-*cis*-βC-ISO2 catalyzed the conversion of 28% of the *all-trans* βC into *9-cis* βC, compared to negligible conversion of the negative control. *9-cis*-βC-ISO1 had a lower but significant activity (not shown).

Activation of the expression of *9-cis-βC-iso*2 in the mutant *E. coli* results in a very significant enhancement of the conversion of *all-trans* βC into *9-cis* βC. The enhancement is similar in extent to that catalyzed by expressed D27 protein from rice (used as a positive control) after 24 h (not shown), and higher after 48h (Fig. 5). Expression of *9-cis-βC-iso*1 results in a smaller, but significant isomerization activity.

Taken together, these results confirm that *9-cis*-βC-ISO2, and possibly also *9-cis*-βC-ISO1, are indeed βC isomerases that catalyze conversion of *all-trans* βC into *9-cis* βC.

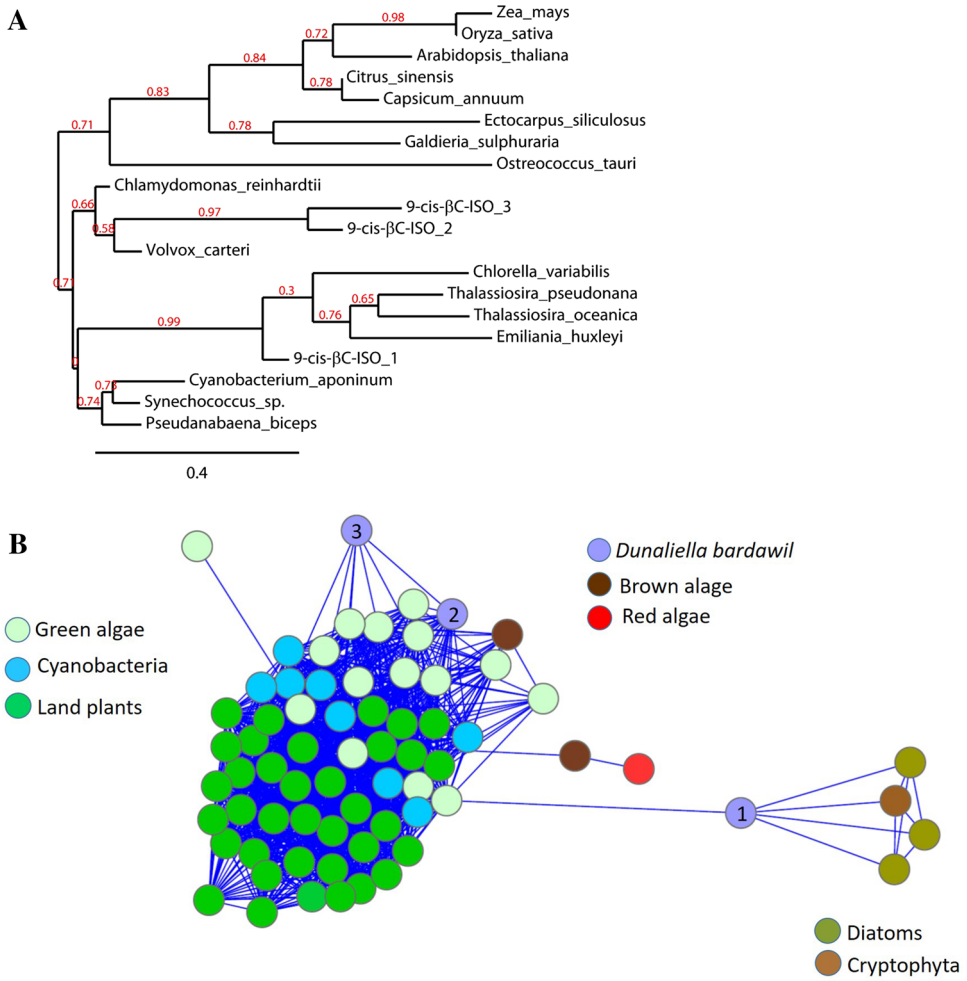

Fig. 2 Phylogenic tree and protein similarity network of *D. bardawil* D-27-like proteins. **a** Phylogenic tree of *D. bardawil* D27-like proteins compared with sequences from other algae, cyanobacteria and plants. Sequence names followed by NCBI accession numbers are as follows: *Oryza sativa* (FJ641055), *Arabidopsis thaliana* (OAP18255.1), *Zea mays* (XP_008670838.1), *Citrus sinensis* (XP_015386200.1), *Capsicum annuum* (XP_016565453.1), *Chlamydomonas reinhardtii* (XP_001697941.1), *Pseudanabaena biceps* (WP_009626659.1), *Volvox carteri* (XP_002949674.1), *Cyanobacterium aponinum* (WP_015220760.1), *Ostreococcus tauri* (XP_003080599.1), *Thalassiosira pseudonana* (XP_002287084.1), *Thalassiosira oceanica* (EJK55179.1), *Synechococcus* sp. (WP_006454034.1), *Chlorella variabilis* (XP_005847526.1), *Galdieria sulphuraria* (XP_005705238.1), *Emiliania huxleyi* (XP_005759170.1) and *Ectocarpus siliculosus* (CBJ33899.1). **b** A protein similarity network was generated from an all-versus-all BLAST analysis (pairwise alignment between all pairs of proteins) of D27 sequences in a local sequence database, using an *E* value of 1e−45. Nodes representing *D. bardawil* are colored *purple* and marked 1–3 for *9-cis*-βC-ISO1 to 3 respectively, land plants (Embryophyte) D27s nodes are colored *dark green*, green algal (Chlorophyceae) D27s nodes are colored *light green*, nodes representing cyanobacterial D27s are colored *light blue*, brown algal D27s colored *dark brown*, red algal nodes are colored *red*, diatom nodes are colored in *olive* and cryptophya node is colored in *light brown*. (Color figure online)

Discussion

Dunaliella bardawil and *D. salina* Teodoresco are the only known organisms that accumulate large amounts of *9-cis* βC. The significance of this pigment for the algae is most probably its high fat solubility in comparison to *all-trans* βC, which allows packaging of very high levels of βC in triacylglycerol lipid globules, amounting to 50% of their weight (Ben Amotz et al. 1982; Jin and Polle 2009). The special significance of *9-cis* βC for humans results from the fact that all mammals need in their diet both *all-trans* and *9-cis* βC, as precursors for *all-trans* and *9-cis* RA, and because *9-cis* βC is present only in small quantities in fruits and vegetables and cannot be chemically synthesized (Schreiber and Carle 2005; Khoo et al. 2011). Therefore, identification of *9-cis* βC isomerases is of special importance and may have an applicative value.

A recent study that addressed the specificity of OsD27 demonstrated high specificity for βC with respect to other carotenoid intermediates such as lycopene, ζ-carotene

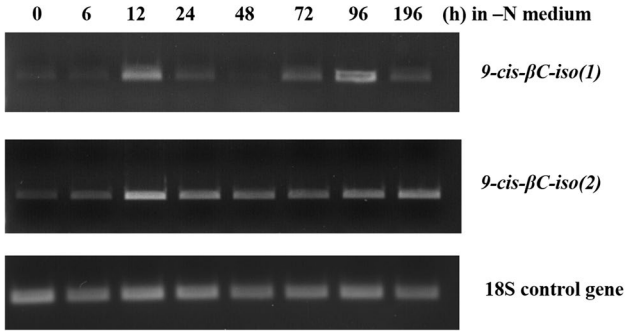

Fig. 3 Induction of mRNA expression during N-deprivation/high light stress. The time course of mRNA expression *9-cis-βC-iso1* and *9-cis-βC-iso2* genes at 0–196 h after onset of N deprivation/high light stress is shown with reference to a control (actin) gene. mRNA level was estimated by semi-quantitative PCR

and zeaxanthin (Bruno and Al-Babili 2016). If *9-cis*-βC-ISO1 and *9-cis*-βC-ISO2 have a similar high specificity to βC, then it seems unlikely that they catalyze the *9-cis* isomerization of phytoene and of subsequent carotenoid intermediates (Shaish et al. 1990), which could explain this discrepancy.

The 9-*cis* βC isomerases in *D. bardawil* show remarkable differences from higher plant D27, both in their function and in their amino acid sequence homology. Whereas in higher plants the role of this enzyme seems to be limited to the synthesis of carlactone, a plant hormone made in very low quantities (Alder et al. 2012), in *D. bardawil* it catalyzes the formation of a major chemical component that amounts to 10% of the dry mass of the cell. However, the wide distribution of D27-like proteins not only in higher plants but also in most algae and even in cyanobacteria (Fig. 2), which do not produce carlactone, suggests that it may be an important family of enzymes that have additional functions. Of the two proteins, *9-cis*-βC-ISO2 more resembles in sequence the majority of higher plants and algal D27-like proteins, whereas *9-cis*-βC-ISO1 seems to belong to a smaller subgroup present mostly in diatoms (Fig. 2). The low amino acid sequence homology probably reflects not just the evolutionary divergence between higher plants and green algae, but also the fact that these enzymes probably evolved late in the evolution both of higher plants and of *Dunaliella*, since they do not catalyze a central and essential metabolic activity. Also the large divergence in structure between the sequences of *9-cis*-βC-ISO1 and *9-cis*-βC-ISO2 suggest that these enzymes diverged long ago during the evolution of this species, without high evolutionary pressures. The remarkable difference in activity between the two enzymes suggests that *9-cis*-βC-ISO2 is the major catalytic contributor to the isomerization reaction and that *9-cis*-βC-ISO1 is either a back-up enzyme or that to achieve full activity it needs to be activated by an internal factor missing in our artificial assay systems.

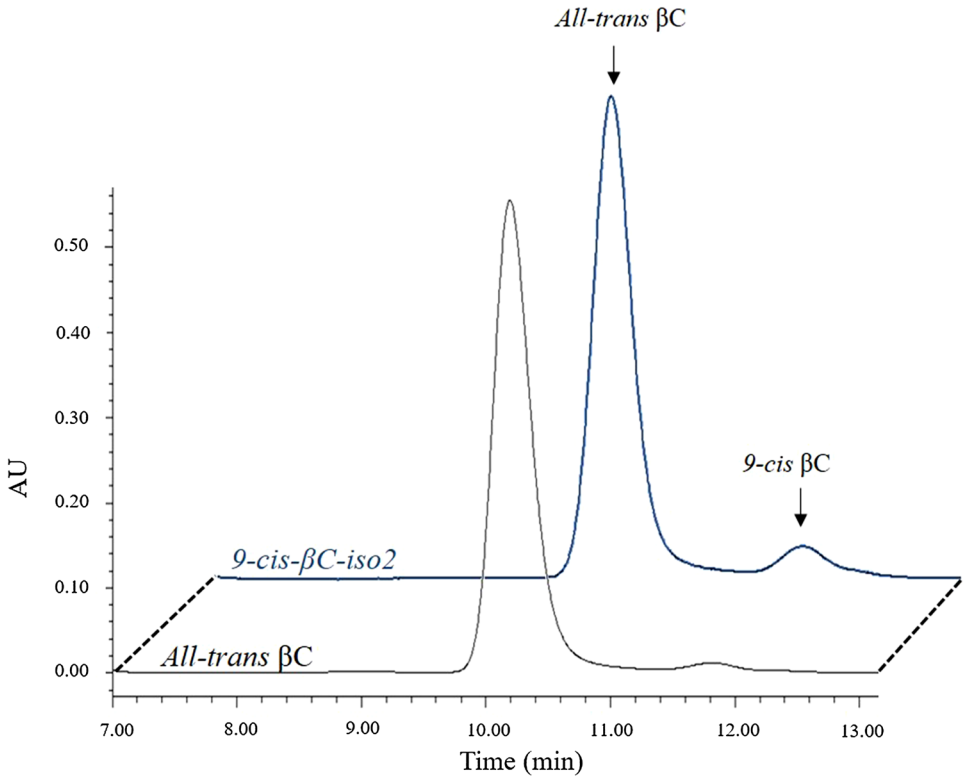

Fig. 4 In vitro activity assay for *9-cis* βC isomerization. Synthetic *9-cis*-βC-ISO2 protein was produced by an *E. coli* mutant as described in "Materials and methods". The purified protein was incubated with 50 µM *all-trans* βC for 2h. Pigments were extracted in *n*-hexane and analyzed on a Halo C8 HPLC column. *Black—all-trans* βC analyzed as control. *Blue*—the product of the in-vitro assay containing *all-trans* βC and peak of *9-Cis* βC peaks. (Color figure online)

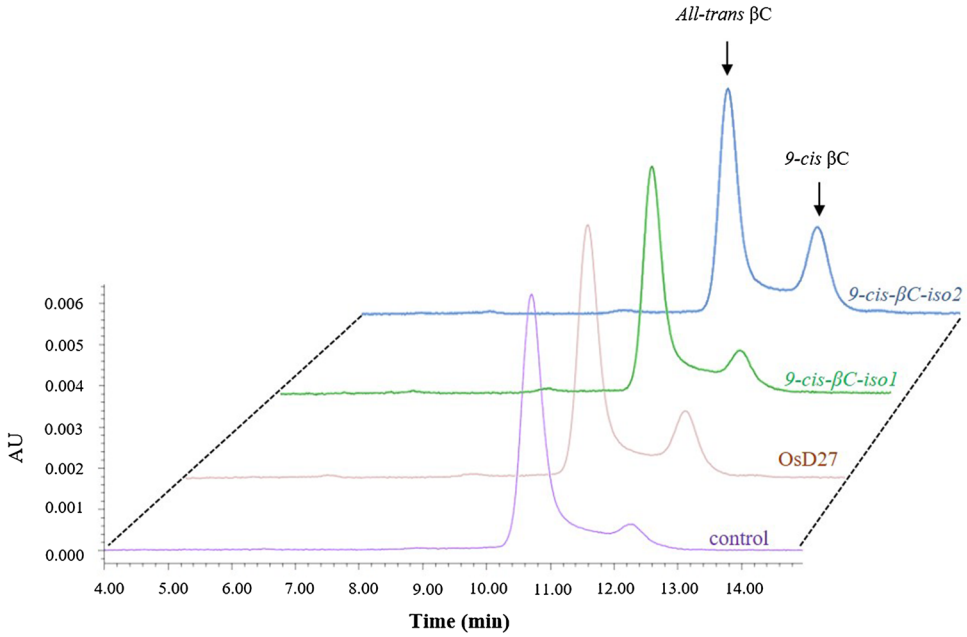

Fig. 5 In vivo activity assay for *9-cis* βC isomerization. *E. coli* mutants, co-expressing βC biosynthesis genes and either *9-cis-βC-iso1* or *9-cis-βC-iso2* or rice *OsD27* genes were cultured for 48 h. Carotenoids were extracted from cell pellets and analyzed on a Halo C8 HPLC column for evaluation of the isomerization from *all-trans* to *9-cis* βC. *Purple*—control *E. coli* having only βC biosynthetic enzymes. *Brown*—*E. coli* co-expressing βC biosynthetic and rice *OsD27*. *Green*—*E. coli* co-expressing βC biosynthetic and *9-cis-βC-iso1*. *Blue*—*E. coli* co-expressing βC biosynthetic and *9-cis-βC-iso2*. (Color figure online)

Author contribution statement The authors thank Dr. Yoav Peleg from the Unit of Structural Proteomics at the Weizmann Institute for his help in synthesis and purification of *9-cis*-βC-ISO1, *9-cis*-βC-ISO2 and rice D27 synthetic proteins. We also thank Prof. Joseph Hirschberg from the Department of Genomics at The Hebrew University in Jerusalem for providing the *E. coli* mutants harboring β-carotene biosynthesis genes. Design of experiments: LD50%, UP50%; experimental work: LD 100%; writing the manuscript: LD 30%, UP 70%

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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