

cDNA cloning of a novel gene codifying for the enzyme lycopene β -cyclase from *Ficus carica* and its expression in *Escherichia coli*

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Abstract Lycopene beta-cyclase (β -LCY) is the key enzyme that modifies the linear lycopene molecule into cyclic β -carotene, an indispensable carotenoid of the photosynthetic apparatus and an important source of vitamin A in human and animal nutrition. Owing to its antioxidant activity, it is commercially used in the cosmetic and pharmaceutical industries, as well as an additive in foodstuffs. Therefore, β -carotene has a large share of the carotenoid market. In this study, we used reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)-PCR to obtain and clone a cDNA copy of the gene *Lyc- β* from *Ficus carica* (*Lyc- β Fc*), which codes for the enzyme lycopene β -cyclase (β -LCY). Expression of this gene in *Escherichia coli* produced a single polypeptide of 56 kDa of weight, containing 496 amino acids, that was able to cycle both ends of the lycopene chain. Amino acid analysis revealed that the protein contained several conserved plant cyclase motifs. β -LCY activity was revealed by heterologous complementation analysis, with lycopene being converted to β -carotene as a result of the enzyme's action. The β -LCY activity of the expressed protein was confirmed by high-performance liquid chromatography (HPLC) identification of the β -carotene. The lycopene to β -carotene conversion rate was 90%. The experiments carried out in this work showed that β -LYC is the enzyme responsible for

converting lycopene, an acyclic carotene, to β -carotene, a bicyclic carotene in *F. carica*. Therefore, by cloning and expressing β -LCY in *E. coli*, we have obtained a new gene for β -carotene production or as part of the biosynthetic pathway of astaxanthin. So far, this is the first and only gene of the carotenoid pathway identified in *F. carica*.

Keywords *Ficus carica* · Lycopene β -cyclase · cDNA · β -Carotene

Introduction

Carotenoids are one of the most diverse and widely distributed groups of natural pigments. They are synthesized by all photosynthetic and many nonphotosynthetic organisms, including bacteria, fungi, algae, and higher plants (D'Ambrosio et al. 2004). They are responsible for the color of fruits and flowers, providing distinct yellow, orange, and red colors, serve as precursors to the plant hormone abscisic acid and thus contributing substantially to plant–animal communication (DellaPenna and Pogson 2006; Hirschberg 2001). In addition, the colors of many carotenoid-accumulating fruits and flowers also contribute to an increase in their economic value (Fraser and Bramley 2004; Rodríguez Concepción 2006).

Carotenoid pigments in the chloroplast of the green tissues function to block the highlight lethal effects of oxygen and free radicals resulting from the transfer of electrons from triplet chlorophyll to molecular oxygen (Frank and Cogdell 1993; Pecker et al. 1996). Carotenoid pigments are synthesized in the isoprenoid biosynthetic pathway within the chloroplasts of plants. Moreover, carotenoids represent essential structural components of the light-harvesting antenna and reaction centre complexes (Horton et al. 1996).

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The biosynthetic pathway of carotenoids is highly complex. The first step is the condensation of two molecules of geranyl geranyl pyrophosphate (GGPP) to originate the C_{40} molecule phytoene (Park et al. 2002). This two-step reaction is catalyzed by phytoene synthase (PSY) a single soluble enzyme (Fig. 1). Two sequential desaturations in the phytoene molecule result in the formation of the first phytofluene and then ζ -carotene. Both of these reactions are carried out by phytoene desaturase (PDS). Two additional desaturations yield the symmetrical red carotenoid pigment lycopene (Britton 1998; Sandmann 1994).

In higher plants, the cyclization of lycopene can occur in two different manners, originating either ϵ -carotene or β -carotene, according to the type of rings on the ends of the molecule (Bouvier et al. 2005; Cunningham 2002). These reactions are catalyzed by two different enzymes ϵ -LCY and β -LCY, respectively. β -LCY acts sequentially on both ends of lycopene, originating β -carotene as a final product and γ -carotene as an intermediate (Cunningham et al. 1994; Hugueney et al. 1995). However, ϵ -LCY can only act on one end of the lycopene molecule, producing δ -carotene (Bouvier et al. 2005; Fraser and Bramley 2004; Hirschberg 2001), which then is converted to α -carotene through a single β -ring addition catalyzed by β -LCY (Cunningham et al. 1996; Hirschberg et al. 1997; Tian et al. 2003) (Fig. 1).

Most carotenoids are produced by chemical synthesis, due to the low productivity of biological systems. But,

because of changing consumer perceptions, primarily in Europe, the natural sources of carotenoids are rapidly gaining economical importance (Rodney 1994). β -Carotene is still the most prominent carotenoid used in foodstuffs and supplements and has the largest share of the carotenoidic market.

Ficus carica L., a deciduous tree belonging to the Moraceae family, is one of the earliest cultivated fruit trees. Today, fig is an important crop worldwide for dry and fresh consumption (Solomon et al. 2006). On the basis of the Dietary Reference Intakes (DRI) data, published by Food and Nutrition Board of the U.S. Institute of Medicine, and the nutrient composition of dried figs (Vinson et al. 2005), they can be demonstrated to be a superior source of minerals, vitamins, amino acids, crude fibers, polyphenols, and several carotenoids, including lycopene, β -carotene, lutein, and α -carotene (Lianju et al. 2003; Vinson 1999; Vinson et al. 2005), with lycopene being the most abundant carotenoid, followed by β -carotene and lutein. Figs contain all major carotenoids appearing in human plasma (Su et al. 2002). However, until now no gene has been described in the biosynthetic pathway of carotenoids in this species.

The purpose of this study was to isolate and clone the full-length cDNA for Lyc- β gene from *F. carica* (Lyc- β Fc), and to analyze the amino acid sequence of this enzyme, as it could prove very useful for futures studies in metabolic engineering to create new biological systems able to produce high levels of natural β -carotene or serve as an intermediary in the path to produce other carotenoids such as xanthophyll astaxanthin.

Materials and methods

Bacteria and plasmids used in this work

Polymerase chain reaction (PCR)-amplified fragments were cloned into the plasmid pCR Blunt II TOPO (Invitrogen), and the DNA constructs obtained were transformed into *Escherichia coli* TOP10 (Invitrogen). Transformed *E. coli* TOP10 cells were grown in LB medium at 37 °C for 12 h. Kanamycin (50 μ g/ml) was employed for selection of transformants.

Plasmid derived from pACYC184 was pACCRT-EIB (Misawa and Shimada 1998), which harbors the *Erwinia uredovora crtE* (GGPP synthase), *crtB* (phytoene synthase), and *crtI* (phytoene desaturase) genes and mediates the formation of lycopene, was a present from Prof. Misawa (Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Japan). This plasmid was transformed into *E. coli* BL21 (DE3) to achieve *E. coli* BL-pACC, a strain of able to produce lycopene and used for color complementation assays (Table 1). Transformed *E.*

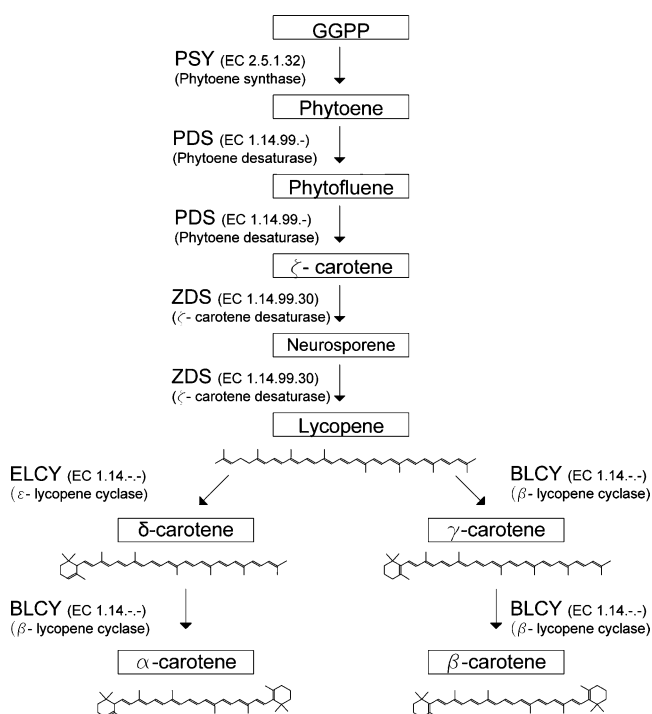


Fig. 1 Schematic diagram of the early steps in the carotenoid biosynthetic pathway in plants

Table 1 Characteristics and carotenoid contents of the *E. coli* transformants used in this study

<i>E. coli</i> strain	Plasmid(s)	Gene(s)	Source	Enzyme common name	Substrates	Product	GenBank accession no.
<i>E. coli</i> BL-pACC	pACCRT-EBI	Eu-crt E	<i>Erwinia uredovora</i>	GGPP synthase	FPP/GPP	GGPP	D90087
		Eu-crt B	<i>Erwinia uredovora</i>	Phytoene synthase	GGPP	Phytoene	D90087
		Eu-crt I	<i>Erwinia uredovora</i>	Phytoene desaturase	Phytoene	Lycopene	D90087
					ζ-carotene	Lycopene	D90087
					Neurosporene	Lycopene	D90087
<i>E. coli</i> BFc-pACC	pET21-Lycβ	Lyc-β Fc	<i>Ficus carica</i>	Lycopene beta-cyclase	Lycopene	β-carotene	JF279547
					γ-carotene	β-carotene	JF279547
					δ-carotene	α-carotene	JF279547
<i>E. coli</i> BLFc-pACC	pACCRT-EBI	Eu-crt E	<i>Erwinia uredovora</i>	GGPP synthase	FPP/GPP	GGPP	D90087
		Eu-crt B	<i>Erwinia uredovora</i>	Phytoene synthase	GGPP	Phytoene	D90087
		Eu-crt I	<i>Erwinia uredovora</i>	Phytoene desaturase	Phytoene	Lycopene	D90087
					ζ-carotene	Lycopene	D90087
					Neurosporene	Lycopene	D90087
	pET21-Lycβ	Lyc-β Fc	<i>Ficus carica</i>	Lycopene beta-cyclase	Lycopene	β-carotene	JF279547
					γ-carotene	β-carotene	JF279547
					δ-carotene	α-carotene	JF279547

coli BL21 (DE3) cells were grown in LB medium at 28 °C for 48 h in the darkness, to maximize carotenoid production. Chloramphenicol (34 µg/ml) was employed for selection of *E. coli* BL-pACC transformants.

The pET21a (Novagen, Cambridge, UK) expression vector contained the complete ORF (open reading frame) of the gene Lyc-β Fc (pET21-Lycβ) was transformed into *E. coli* BL21 (DE3) (Invitrogen) to achieve *E. coli* BFc-pACC (Table 1). Transformed *E. coli* BL21 (DE3) cells were grown in LB medium at 28 °C in the presence of ampicillin (50 µg/ml) for 48 h when β-LCY enzyme expression was anticipated. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added (1 mM) at the end of the logarithmic growth phase.

Cloning of the lycopene β-cyclase gene from *F. carica*

Total RNA was isolated from 60 mg of fresh leaf from *F. carica*, using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), as recommended by the manufacturer.

The first cDNA strand was synthesized using the reverse transcription (RT)-PCR AMV kit (Roche Applied Science, Indianapolis, IN, USA). Degenerate primers Lyc-β Deg F and Lyc-β Deg R (Table 1), used to carry out RT-PCR, were designed using Primer Premier 5.0 (Biosoft International), according to conserved motifs of other Lyc-β sequences deposited in the GenBank Database. Specific cDNA amplification was carried out by PCR using the above-mentioned oligonucleotide primers and 2 µl of first-strand cDNA. The reaction mixture contained polymerase buffer, 0.2 mM of each primer, 1.5 mM MgCl₂, 0.2 mM of each

deoxynucleotide, and 1 unit of Accuzyme DNA polymerase (Bioline, Taunton, MA).

PCR reactions to amplify the internal fragment of Lyc-β Fc were carried out using the following program: 94 °C for 2 min, 35 cycles of 94 °C for 45 s; 49 °C for 1 min and 72 °C for 2 min; and a final extension of 72 °C for 2 min. The PCR product was cloned into the pCR Blunt II TOPO vector (Invitrogen) using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). DNA inserts were then sequenced.

RACE-PCR

The 5' and 3' ends of Lyc-β Fc were obtained by rapid amplification of cDNA ends (RACE)-PCR, using the 5'/3' RACE kit, 2nd Generation (Roche Applied Science). The primers Lyc-β Fc1, Lyc-β Fc2, Lyc-β Fc3, Lyc-β Fc4, and Lyc-β Fc5 (Table 2) were designed for the RACE-PCR. The amplification was carried out with 1 unit of Accuzyme DNA Polymerase. Lyc-β Fc1 was the primer used for first strand cDNA synthesis. The missing 5' region of the gene was obtained in a PCR containing Lyc-β Fc2 and Oligo dT-Anchor primer as primers, and incubated as follows: 1 cycle at 94 °C for 2 min; 10 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 40 s; 25 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 40 s and increasing the elongation time by 20 s in each subsequent cycle; and a final extension cycle of 72 °C for 7 min. The resulting DNA product was then used as the template (dilution 1:20) in a subsequent PCR, with Lyc-β Fc3 and the PCR anchor primer as primers. This second PCR was incubated as described above, except for the primer annealing step that was increased to 60 °C.

Table 2 PCR primers used in this study (sequence is written 5' to 3')

Technique	Primer	Primer sequence
RT-PCR	Lyc- β DegF	5' TGGCCHAAAYAATTAYGGWGTGG 3'
RT-PCR	Lyc- β DegR	5' ATATCCATDCCAAAASAGWAG 3'
5' RACE-PCR	Lyc- β SP1	5' TCCCACCCATTGCCATCACACTT 3'
5' RACE-PCR	Lyc- β SP2	5' GGTGACCCTCAACTTCAGCCAAAA 3'
5' RACE-PCR	Lyc- β SP3	5' GCCTCTCCCTATTGACACGCCCAT 3'
3' RACE-PCR	Lyc- β SP4	5' CGGGGTACCCCGGGGAAATGAGCCTTACTTGCGAG 3'
3' RACE-PCR	Lyc- β SP5	5' CGGGGTACCCCGTGGAGGTGAAGAAGAGGATGGTTGC 3'
PCR (ORF)	Lyc- β <i>NheI</i> F	5' GGAATTCCATATGGGTACTCTTCCATATCCA 3'
PCR (ORF)	Lyc- β <i>SacI</i> R	5' CGAGCTCCTAAATGGTTTCAAGTGCCAAAT 3'

H = A, C; Y = C, T; W = A, T; D = A, G; S = C, G

F forward, R reverse, ORF open reading frame

To obtain the 3' end of Lyc- β Fc, we carried out two sequential PCR reactions, using the Lyc- β Fc4 and Lyc- β Fc5 primers, respectively, and the PCR anchor primer. The DNA fragments obtained were then sequenced.

From the sequences obtained above, we designed PCR primers Lyc- β *NdeI* F and Lyc- β *SacI* R to amplify the complete gene Lyc- β Fc coding region. We added restriction sites *NdeI* and *SacI*, respectively, (Table 2) at the 5' and 3' end of the primers to facilitate future DNA cloning. The PCR conditions were: 1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 54 °C for 60 s and 72 °C for 2 min; and a final extension at 72 °C for 10 min. The resulting PCR product was cloned into a pET21a vector, thus obtaining the expression plasmid pET21-Lyc β (Table 1).

Determination of lycopene cyclase enzymatic activity

The lycopene cyclase enzyme activity was determined by color complementation assays using the strain *E. coli* BL-pACC as a start point (Table 1). This strain displays a red coloration due to lycopene accumulation.

E. coli BL-pACC was co-transformed with the plasmid pET21-Lyc β to achieve *E. coli* BLFc-pACC. This strain displays an orange coloration due to the accumulation of specific carotenoid, providing an easily screenable phenotype. Co-transformed cells were plated on LB agar plates containing chloramphenicol (34 μ g/ml) and ampicillin (50 μ g/ml), and spread with 10 μ l of a 100 mM aqueous solution of Isopropylthio- β -D-galactoside (IPTG) 1 h before the cells were plated. The plates were incubated at 28 °C for 48 h in the darkness, to maximize carotenoid production. *E. coli* BL-pACC transformed with the plasmid pET21a was used as the negative control. After incubation, the cyclase activity on the lycopene molecule was identified by the color of the bacterial colonies; a red color meant no cyclase activity, whereas an orange/yellow resulted from the enzymatic activity.

HPLC analysis of carotenoids

E. coli BLFc-pACC (Table 1) was grown over night in LB broth supplemented with chloramphenicol (34 μ g/ml) and ampicillin (50 μ g/ml) at 37 °C with agitation (200 rpm). The culture was then incubated for another 48 h at 28 °C in the darkness with agitation (200 rpm) after adding isopropyl- β -D-thiogalactopyranoside (1 mM). After incubation, the cells were centrifuged (4,000 $\times$ g for 5 min), and the bacterial pellet washed twice with deionized water, re-suspended in acetone (Sigma) and homogenized by vortexing (10 min at 4 °C). After a new centrifugation (13,000 $\times$ g, 2 min, 4 °C), the supernatant was recovered and dried down under a N₂ flow, and kept at -80 °C until high-performance liquid chromatography (HPLC) analysis. All operations were carried out on ice under dim light to prevent photo-degradation, isomerizations, and structural changes of the carotenoids. The samples were prepared for HPLC by dissolving the dried residues in 2 ml of chloroform/methanol/acetone (3:2:1 v/v/v) and filtered through polycarbonate 0.22- μ m filter. The HPLC device used was equipped with a photodiode array detector and was controlled by the Empower2 software program. A C₃₀ carotenoid column (250 $\times$ 4.6 mm, 5 μ m; YMC Europa) was used, and the mobile phase was as follows: A, methyl *tert*-butyl ether; B, water; C, methanol. The linear ternary gradient elution program was performed as follows: initially, A/B/C (5:5:90); then 0–12 min, A/B/C (5:0:95); 12–20 min, A/B/C (14:0:86); 20–30 min, A/B/C (25:0:75); 30–50 min, A/B/C (50:0:50); 50–70 min, A/B/C (75:0:25); and finally back to A/B/C (5:5:90) for re-equilibration. The flow rate was maintained at 1 ml/min, the temperature at 24 °C and the sample volume was 20 μ l. Carotenoids were identified by comparing their HPLC retention time and color with standard compounds or with reported data. The β -carotene and lycopene standards were obtained

from Sigma-Aldrich (Madrid, Spain) as described (Melendez Martinez et al. 2003; Rodrigo et al. 2004). The photodiode array detector was set to scan from 250 to 540 nm throughout all of the elution profile. For each elution, a Maxplot chromatogram was obtained, which plots each carotenoid peak at its corresponding maximum absorbance wavelength. Quantitative analysis was done by calculating peak areas of the chromatogram using a calibration curve obtained with a β -carotene standard.

Protein electrophoresis (SDS-PAGE)

E. coli BL21 (DE3) transformed with either pET21a or pET21-Lyc β was grown at 37 °C, with agitation (180 rpm), until it reached an OD of 0.5–1. The cultures were then induced by addition of 1 mM IPTG. After four hours incubation, samples were taken and according to the instructions provide in the pET21a system manual (Novagen). Prior to polyacrylamide gel electrophoresis, the samples were incubated at 85 °C for 3 min in solubilization buffer containing 2.5% (w/v) SDS, 125 mM dithiothreitol, 25% (v/v) glycerol and 112.5 mM Tris-HCl, pH 6.8. The protein samples were then loaded onto a 12% polyacrylamide gel and separated on a Mini Protean II cell (Bio-Rad, Hercules, CA), according to the method of Laemmli (1970). The gel was run at 120 V for 1 h, using Tris-Glycine buffer (25 mM Tris; 192 mM Glycine; 0.1% SDS; pH: 8.3). The molecular weight of the proteins was determined using Precision Plus Protein Standard (Bio-Rad) and the software Quantity One (Bio-Rad).

Bioinformatic analyses

Vector NTI Advance 9.0 software (Invitrogen) and BioEdit Sequence Alignment Editor version 7.0.5.3, were used to analyze the nucleotide and deduced amino acid sequences, and for sequence alignment, respectively. The NCBI database was searched for plant β -LYC sequences using the BLAST software (Altschul et al. 1990).

The ChloroP 1.1 Prediction Server program (Emanuelsson et al. 1999) was used to identify the β -LCY signal/sorting peptide. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 package program (Kumar et al. 2004). Data were analyzed by neighbor-joining method. The reliability of the neighbor-joining tree (Saitou and Nei 1987) was estimated by calculating bootstrap confidence limits (BCL) (Peelman et al. 2003) based on 1,000 replicates. The Gen Bank accession numbers of the nucleotides used in the analysis are shown in Fig. 4. Subcellular sorting was predicted by PSORT Web server (Nakai and Horton 1999) for analyzing and predicting protein-sorting signals at the Institute for Molecular and Cellular Biology (Osaka, Japan). Finally, PSIPred v3.0 (Jones 1999), was used for hydrophobicity and for protein secondary structure predictions.

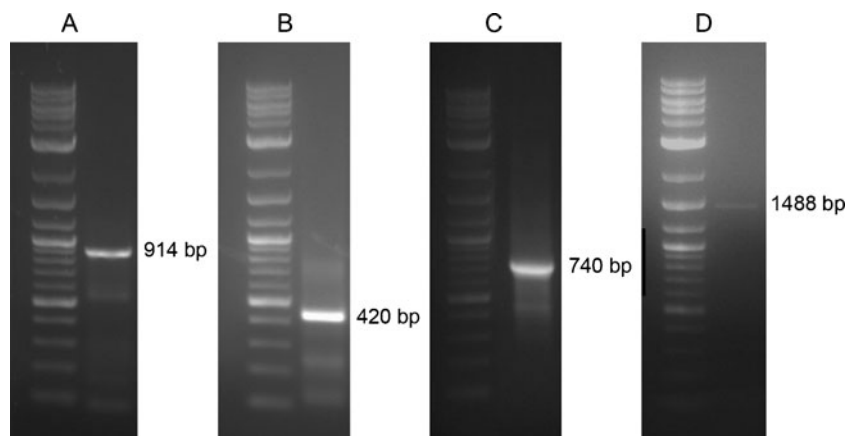
Results

Lyc- β Fc cDNA sequence

We obtained, by RT-PCR, a single 914 bp PCR product using the conserved sequences-based primers Lyc- β Deg F and Lyc- β Deg R (Fig. 2a). By sequence analyses, the amplicon displayed a high similarity (74–77%) to other plant lycopene-cyclase genes, such as those from *Citrus sinensis* (ABB72443), *Actinidia deliciosa* (FJ427509), *Actinidia chinensis* (FJ427508), *Lycopersicon esculentum* (X86452), and *Carica papaya* (ABD91578).

We had to resort to RACE-PCR to obtain the sequences of the 5' and 3' ends of Lyc- β Fc. The 5' end of the gene was amplified as a 420-bp fragment (Fig. 2b), whereas a fragment of 750 bp (Fig. 2c) represented the 3' end of the gene. Both new sequences overlapped the known internal sequence of the gene. Finally, the complete sequence of the Lyc- β Fc cDNA from *F. carica* could be determined (JF279547). The complete ORF **of the gene had a length

Fig. 2 Isolation of the full-length Lyc- β cDNA from *Ficus carica*. **a** Fragment of the Lyc- β Fc cDNA internal sequence, amplified with the primer pair Lyc- β DegF and Lyc- β DegR. **b** The 5' end of the Lyc- β Fc cDNA, isolated by 5'-RACE. **c** The 3' end of the Lyc- β Fc cDNA, isolated by 3'-RACE. **d** Open reading frame of the full-length Lyc- β Fc cDNA from *F. carica*



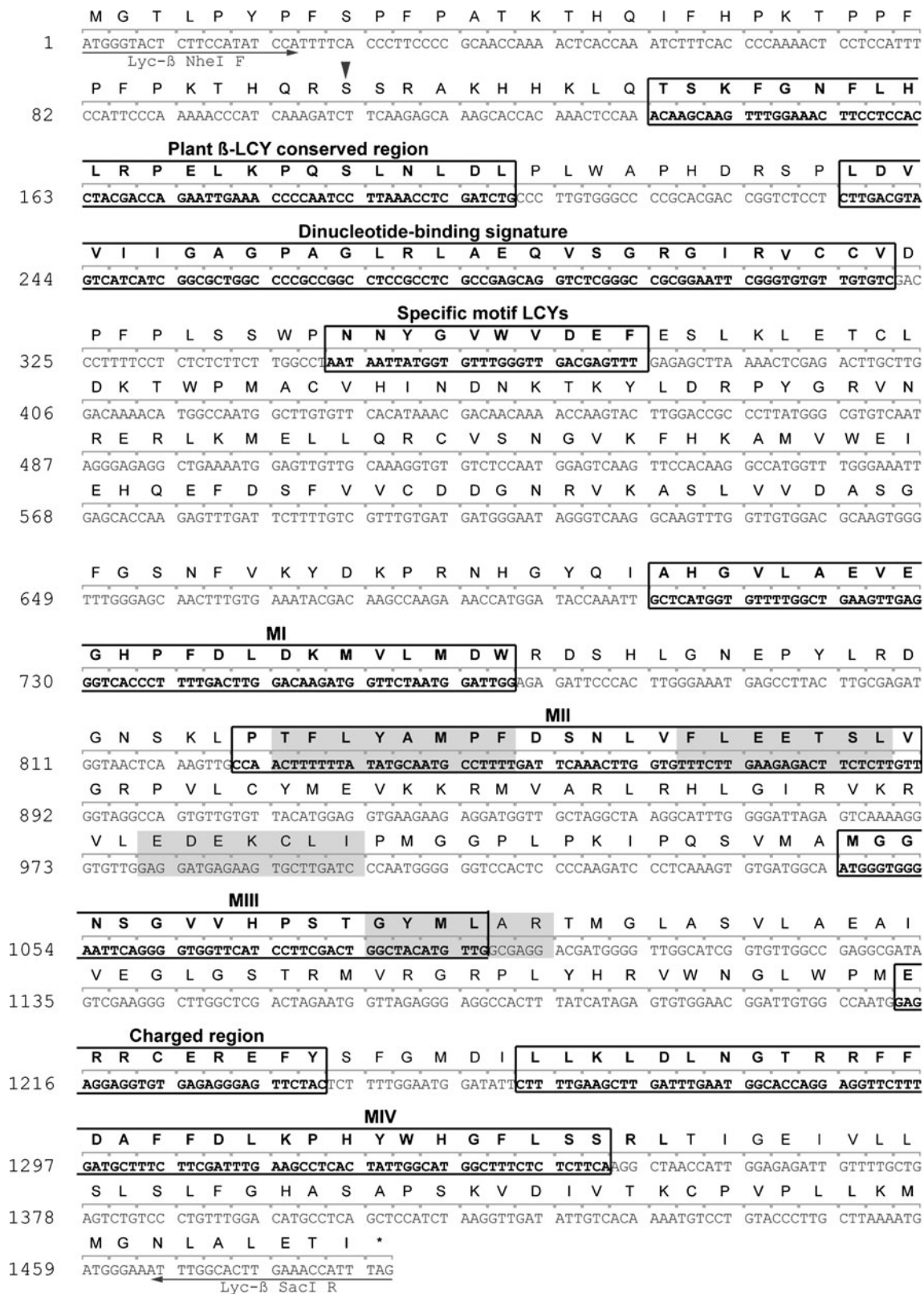


Fig. 3 Characteristics of the *F. carica* beta lycopene cyclase protein. The most likely points for chloroplast precursor cleavage are indicated with an arrow. Characteristic regions of the plant LCYs are indicated by open boxes, the dinucleotide binding signature, cyclase motifs (M)

I to IV, and charged region (Cunningham et al. 1996; Hugueney et al. 1995). Domains described as essential for β-LCY activity are boxed in gray (Bouvier et al. 1997)

of 1,488 bp (Fig. 2d), and the predicted amino acid sequence specified a polypeptide of 496 amino acids in length. This was the amplicon co-expressed in *E. coli* BL-pACC.

Several featured structures of interest were found when the predicted *F. carica* Lyc- β Fc sequence was compared and aligned with other lycopene β -cyclases (Fig. 3). Data analysis, with the PSORT program, predicted a plastid location for the β -LCY protein, with a predicted transit peptide of 36 bp (Fig. 3). β -LCY from *F. carica* contains the specific highly conserved LCY motif, characteristic of this kind of enzymes in higher plants. The analysis of conserved motifs along the amino acid sequences showed the presence of a dinucleotide-binding signature, typically DX₄GXXAX₄A, with a secondary structure of β sheet– α -helix loop– β sheet. This motif is referred to as the “dinucleotide binding fold” that is present in all plant β -LCYs (Cunningham et al. 1994; Wirenga et al. 1986) (Fig. 3). Other than that, the rest of the essential motifs for cyclase activity were present in the β -LCY of *F. carica* (Bouvier et al. 1997; Cunningham et al. 1996).

β -LYC Fc expression and characterization

When the *E. coli* BL21 (DE3)-expressed pET21-Lyc β was analyzed by SDS/PAGE, a protein of 56 kDa of molecular weight could be observed. This band only appeared in the cells expressing the construct and after the induction of the plasmid with IPTG (1 mM), but not in the negative controls. However, studies were not performed on tissue differential expression or at different stages of plant development.

The Neighbor-Joining test showed a high homology of the expressed protein with lycopene β -cyclase genes of plants. The marine microalgae *Dunaliella salina* was used as an outgroup and appears in an isolated branch. The position of the sequence from *F. carica* was more closely related to *A. deliciosa* and *A. chinensis* than to other β -LCYs so far examined (Fig. 4).

Color complementation assays were used to analyze the enzymatic activity of β -LCY. These tests are based on the color differences between the carotenoids; lycopene is red, whereas β -carotene is orange/yellow. When *E. coli* BL-pACC was co-transformed with pET21-Lyc β , and β -LCY expression induced, the color of the bacterial colonies turned from red to orange/yellow, thus indicating a variation in the carotenoid composition from lycopene to β -carotene (Fig. 5). The negative controls did not show changes in their colors.

Carotenoid identification and quantification by HPLC

The analyses of carotenoids present in the negative controls showed that the initial pigment in the cells was lycopene, whereas the pigment after β -LCY expression of *E. coli* BLFc-pACC corresponded to β -carotene. This strongly suggested the ability of β -LCY to act on both ends of the

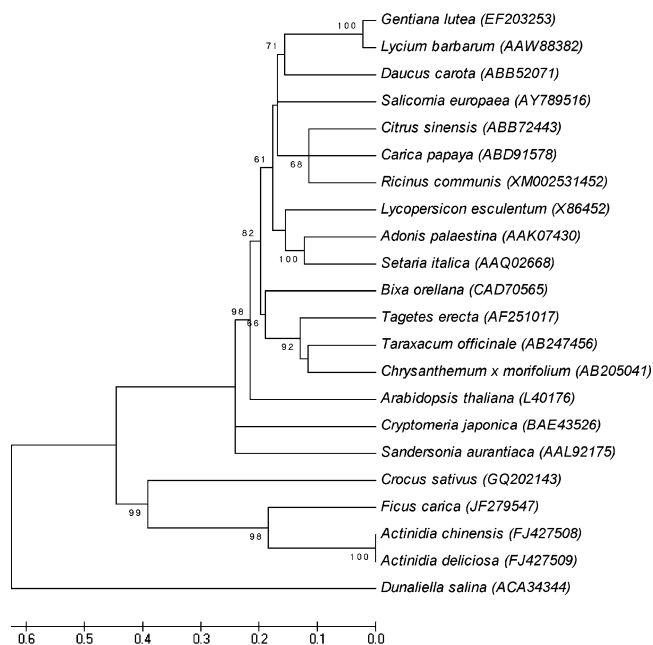


Fig. 4 Phylogenetic tree generated based on alignment of nucleotide sequence of different β -LCYs. The tree was inferred using the Neighbor-Joining method. The Bootstrap values on the nodes indicate the number of times that each group occurred with 1,000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). Accession numbers of nucleotide sequences are in parenthesis

lycopene molecule as expected from a β -cyclase activity. We did not observe accumulation of the monocyclic species γ -carotene in cultures of *E. coli* BLFc-pACC. This is consistent with studies conducted so far, claiming that all known β -cyclases, except one recently discovered in a marine bacterium (Teramoto et al. 2003), create rings at both ends of lycopene to form β -carotene via γ -carotene (Daisuke et al. 2005; Sandmann et al. 1990).

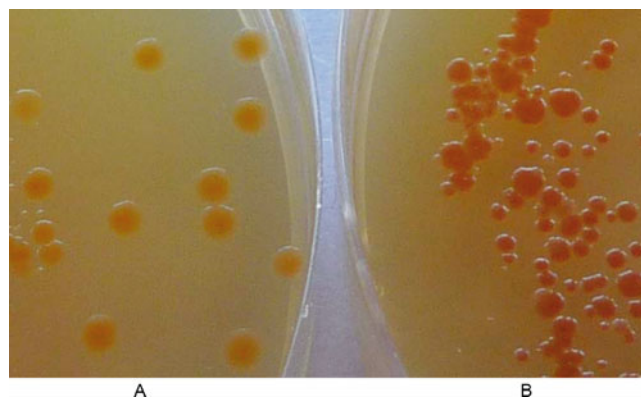


Fig. 5 Complementation color analysis. **a** *E. coli* BL-pACC transformed with pET21-Lyc β . **b** Negative control: *E. coli* BL-pACC expressing pET21a, without Lyc- β gene

The β -carotene content of the recombinant *E. coli* BLFc-pACC was $529 \mu\text{g/g}^{-1}$ of dry weight cells and the β -cyclase activity, defined as the percentage of lycopene/ β -carotene conversion rate, was 91%. These results show higher values of beta carotene production compared with those obtained in other recombinant *E. coli* strains (Misawa et al. 1995; Misawa and Shimada 1998; Schurr et al. 1996), but lower than other *E. coli* strains carrying the exogenous *ipi* gene for IPP isomerase (Misawa and Shimada 1998).

In addition, we observed that *E. coli* BLFc-pACC transformant varies for level of pigmentation. When grown on plates at 28 °C in the darkness, it showed the highest accumulation of pigment.

Discussion

β -Carotene is still the most prominent carotenoid on the market; however, most carotenoids are still produced by chemical synthesis, due to the low productivity of the biological systems (Rodney 1994). One way to increase the productivity of carotenoid synthesis would be the use of recombinant DNA technology, but before this technology can be used, the carotenoid biosynthetic genes need to be isolated and characterized.

In this study, we were able to clone and identify the complete sequence encoding the lycopene cyclase enzyme from *F. carica*, and to carry out its heterologous expression in *E. coli*. The molecular mass of the newly expressed protein, as isolated from the soluble cytoplasmic fraction, was 56 kDa, which corresponded to the expected mass calculated from the gene sequence.

The amino acid sequence of the ORF, displayed high similarity with the β -LCYs from other plants. Thus, the presence in the N-terminal end of the signal peptide for plastid importation is typical of all the enzymes involved in the biosynthesis of carotenoids in plants (Bouvier et al. 2005; Fraser and Bramley 2004; Sandmann et al. 2006) and, as expected, is not present in the β -LCYs from bacteria or cyanobacteria (Hugueney et al. 1995). Within this region, it was possible to identify the “Plant β -LCY conserved region,” that has been proposed as essential for the association of β -LYC to the membrane and for its catalytic activity (Beyer et al. 1991; Hugueney et al. 1995).

Lycopene is the primary substrate for the formation of cyclic carotenoids in plants. The conversion of lycopene to β -carotene requires that a cyclization reaction occurs at both ends of the symmetrical lycopene. The analysis of carotenoids showed the presence of β -carotene, and we were unable to observe accumulation of the monocyclic γ -carotene in cultures containing pACCRT-EIB and pET21-Lyc β , even though a substantial amount of lycopene

accumulated in these cells. This observation indicates that any molecule of lycopene which is cyclized at one end has high probability of being cyclized at the other end as well. The possibility that lycopene cyclase operates as a homodimeric complex would be one explanation for this result (Gallagher et al. 2003). Our result demonstrated that a single plant enzyme efficiently catalyzes sequentially both cyclization steps like other plants β -LCYs described so far (Ahrazem et al. 2009; Alquezar et al. 2009; Ampomah-Dwamena et al. 2009; Hugueney et al. 1995; Rodrigo et al. 2004).

On the other hand, the heterologous expression of biosynthetic pathways in *E. coli* continues to be a powerful approach for developing metabolic engineering applications in plants. The utility of the bacterial system lies in its inherent similarity to the biochemistry of the plant plastid (Gallagher et al. 2003).

It can therefore be concluded that the Lyc- β Fc gene encodes for a lycopene β -cyclase, capable of synthesizing β -carotene, a pigment of high industrial interest and of great importance in health and human and animal nutrition.

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References

- Ahrazem O, Rubio-Moraga A, Castillo R, Gomez-Gomez L (2009) The expression of chromoplast-specific lycopene beta cyclase gene is involved in the high production of saffron's apocarotenoid precursors. *J Exp Bot* 61(1):105–119. doi:10.1093/jxb/erp283
- Alquezar B, Zacarias L, Rodrigo MJ (2009) Molecular and functional characterization of a novel chromoplast-specific lycopene β -cyclase from *Citrus* and its relation to lycopene accumulation. *J Exp Bot* 60(6):1783–1797. doi:10.1093/jxb/erp048
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215(3):403–410
- Ampomah-Dwamena C, McGhile T, Wibisono R, Montefiori M, Hellens RP, Allan AC (2009) The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit. *J Exp Bot* 60(13):3765–3779. doi:10.1093/jxb/erp218
- Beyer P, Kröncke U, Nievelstein V (1991) On the mechanism of the lycopene isomerase/cyclase reaction in *Narcissus pseudonarcissus* L. chromoplasts. *J Biol Chem* 266(26):17072–17078
- Bouvier F, d'Harlingue A, Camara B (1997) Molecular analysis of carotenoid cyclase inhibition. *Arch Biochem Biophys* 346(1):53–64
- Bouvier F, Rahier A, Camara B (2005) Biogenesis, molecular regulation and function of plant isoprenoids. *Prog Lipid Res* 44(6):357–429
- Britton G (1998) Overview of carotenoid biosynthesis. In: Britton G, Liaaen-Jensen S and Pfander H (eds) *Carotenoids: biosynthesis and metabolism*. Birkhäuser Verlag, Basel, Switzerland, pp 13–147
- Cunningham FX (2002) Regulation of carotenoid synthesis and accumulation in plants. *Pure Appl Chem* 74:1409–1417

- Cunningham FX, Sun Z, Chamovitz D, Hirschberg J, Gantt E (1994) Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp. strain PCC7942. *Plant Cell* 6:1107–1121
- Cunningham FX, Pogson B, Sun Z, McDonald KA, DellaPenna D, Gantt E (1996) Functional analysis of the β and ϵ lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell* 8(9):1613–1626
- D'Ambrosio C, Giorio G, Marino I, Merendino A, Petrosz A, Salfi L, Stigliani A, Cellini F (2004) Virtually complete conversion of lycopene into beta-carotene in fruits of tomato plants transformed with the tomato lycopene beta cyclase (tlcy-b) cDNA. *Plant Sci* 166:207–214. doi:10.1016/j.plantsci.2003.09.015
- Daisuke U, Alexander VT, Frances HA (2005) Diversifying carotenoid biosynthetic pathways by directed evolution. *Microbiol Mol Biol Rev* 69(1):51–78. doi:10.1128/MMBR.69.1.51-78.2005
- DellaPenna D, Pogson BJ (2006) Vitamin synthesis in plants: tocopherols and carotenoids. *Annu Rev Plant Biol* 57(1):711–738
- Emanuelsson O, Nielsen H, Von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci* 8(5):978–984
- Frank H, Cogdell RJ (1993) Photochemistry and function of carotenoids in photosynthesis. In: Young AJ, Britton G (eds) *Carotenoids and photosynthesis*. Chapman & Hall, London, pp 253–326
- Fraser PD, Bramley P (2004) The biosynthesis and nutritional uses of carotenoids. *Prog Lipid Res* 43(3):228–265
- Gallagher CE, Cervantes M, Wurtzel ET (2003) Surrogate biochemistry: use of *Escherichia coli* to identify plant cDNAs that impact metabolic engineering of carotenoid accumulation. *Appl Microbiol Biotechnol* 60(6):713–719
- Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. *Curr Opin Plant Biol* 4(3):210–218
- Hirschberg J, Cohen M, Harker M, Lotan T, Mann V, Pecker I (1997) Molecular genetics of the carotenoid biosynthesis pathway in plants and algae. *Pure Appl Chem* 69(10):2151–2158
- Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. *Annu Rev Plant Physiol Plant Mol Biol* 47:655–684
- Hugueney P, Badillo A, Chen HC, Klein A, Hirschberg J, Camara B, Kuntz M (1995) Metabolism of cyclic carotenoids: a model for the alteration of this biosynthetic pathway in *Capsicum annuum* chromoplasts. *Plant J* 8(3):417–424
- Jones DT (1999) Protein secondary structure prediction based on position-specific scoring matrices. *J Mol Biol* 292:195–202
- Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings Bioinf* 5:150–163
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London, UK)* 227:680–685
- Lianju W, Weibin J, Kai M, Zhifeng L, Yelin W (2003) The production and research of fig (*Ficus carica* L.) in China. *Acta Horticult* 605:191–196
- Melendez Martinez AJ, Vicario IM, Heredia FJ (2003) A routine high-performance liquid chromatography method for carotenoid determination in ultrafrozen orange juices. *J Agric Food Chem* 51:4219–4224
- Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiura S, Saito T, Ohtanai T, Miki W (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J Bacteriol* 177:6575–6584
- Misawa N, Shimada H (1998) Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. *J Biotechnol* 59(3):169–181
- Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci* 24(1):34–35
- Park H, Kreunen SS, Cuttriss AJ, DellaPenna D, Pogson BJ (2002) Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation and photomorphogenesis. *Plant Cell* 14:321–332
- Pecker I, Gabbay R, Cunningham FX, Hirschberg J (1996) Cloning and characterization of the cDNA for lycopene beta-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Mol Biol* 30:807–819
- Peelman F, Labeur C, Vanloo B, Roosbeek S, Devaud C, Duverger N, Deneffe P, Rosier M, Vandekerckhove J, Rosseneu M (2003) Characterization of the ABCA transporter subfamily: identification of prokaryotic and eukaryotic members, phylogeny and topology. *J Mol Biol* 325:259–274
- Rodney AL (1994) Production of carotenoids by recombinant DNA technology. *Pure Appl Chem* 5(66):1057–1062
- Rodrigo MJ, Marcos JF, Zacarias L (2004) Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. *J Agric Food Chem* 52(22):6724–6731
- Rodríguez Concepción M (2006) Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. *Phytochem Rev* 1(5):1–15
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sandmann G (1994) Phytoene desaturase — genes, enzymes and phylogenetic aspects. *J Plant Physiol* 143(4–5):444–447
- Sandmann G, Woods WS, Tuveson RW (1990) Identification of carotenoids in *Erwinia herbicola* and in a transformed *Escherichia coli* strain. *FEMS Microbiol Lett* 59(1–2):77–82
- Sandmann G, Romer S, Fraser PD (2006) Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab Eng* 8(4):291–302
- Schurr G, Misawa N, Sandmann G (1996) Expression, purification and properties of lycopene cyclase from *Erwinia uredovora*. *Biochem J* 315:869–874
- Solomon A, Golubowicz S, Yablowicz Z, Grossman S, Bergman M, Gottlieb HE, Altman A, Kerem Z, Flaishman MA (2006) Antioxidant activities and anthocyanin content of fresh fruits of common fig (*Ficus carica* L.). *J Agric Food Chem* 54:7717–7723
- Su Q, Rowley KG, Itsiopoulos C, O'Dea K (2002) Identification and quantitation of major carotenoids in selected components of the Mediterranean diet: green leafy vegetables, figs and olive oil. *Eur J Clin Nutr* 56:1149–1154
- Teramoto M, Takaichi S, Inomata Y, Ikenaga H, Misawa N (2003) Structural and functional analysis of a lycopene beta-monocyclase gene isolated from a unique marine bacterium that produces myxol. *FEBS Lett* 545:120–126
- Tian L, Magallanes-Lundback M, Musetti V, DellaPenna D (2003) Functional analysis of β - and ϵ -ring carotenoid hydroxylases in *Arabidopsis*. *Plant Cell* 15:1320–1332
- Vinson JA (1999) The functional food properties of figs. *Cereal Foods World* 4:82–87
- Vinson JA, Zubik L, Bose P, Samman N, Proch J (2005) Dried fruits: excellent *in vitro* and *in vivo* antioxidants. *J Am Coll Nutr* 24:44–50
- Wirenga RK, Terpstra P, Hol WG (1986) Prediction of the occurrence of the ADP-binding $\beta\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J Mol Biol* 187:101–107