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RESEARCH PAPER

Molecular and functional characterization of a novel chromoplast-specific lycopene β-cyclase from *Citrus* and its relation to lycopene accumulation

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Abstract

Carotenoids are the main pigments responsible of the colouration of *Citrus* fruits. The β-cyclization of lycopene, catalysed by the lycopene β-cyclases (β-LCY), seems to be a key regulatory step of the carotenoid pathway. In the present study, two β-LCYs from orange fruits (*Citrus sinensis*), named *Csβ-LCY1* and *Csβ-LCY2* have been isolated and the activity of the encoded proteins was demonstrated by functional analysis. *Csβ-LCY1* was expressed at low levels and remained relatively constant during fruit ripening while Csβ-*LCY2* showed a chromoplast-specific expression and a marked induction in both peel and pulp of orange fruits in parallel with the accumulation of β,β-xanthophylls. The potential involvement of *Csβ-LCY2* in the accumulation of lycopene, characteristic of some *Citrus* species such as red grapefruits, was investigated. Expression of *Csβ-LCY2* and another seven carotenoid biosynthetic genes were studied in the peel and pulp of the high lycopene-accumulating grapefruit, Star Ruby, and compared with those of ordinary Navel orange. In Star Ruby, the accumulation of lycopene during fruit maturation was associated with a substantial reduction in the expression of both β-*LCY2* and β-*CHX* genes with respect to Navel orange. Moreover, two different alleles of β-*LCY2*: β-*LCY2a* and β-*LCY2b* were isolated from both genotypes, and functional assays demonstrated that the lycopene β-cyclase activity of the allele *b* was almost null. Interestingly, Star Ruby grapefruit predominantly expressed the unfunctional β-*LCY2b* allele during fruit ripening whereas Navel oranges preferably expressed the functional allele. It is suggested that the presence of diverse alleles of the β-*LCY2* gene, encoding enzymes with altered activity, with different transcript accumulation may be an additional regulatory mechanism of carotenoid synthesis involved in the accumulation of lycopene in red grapefruits.

Key words: Carotenoids, *Citrus* fruit, gene expression, grapefruit, lycopene, lycopene β-cyclase, orange fruit.

Introduction

Carotenoids form an important family of isoprenoid pigments synthesized by plants and certain algae, bacteria, and fungi. In photosynthetic organisms carotenoids play essential functions as components of the light-harvesting system, and protecting plant cells against an oxidation-derived excess of light energy (Demmig-Adams *et al.*, 1996). Moreover, *cis*-epoxycarotenoids are the precursors of the plant hormone abscisic acid which plays a crucial role in several physiological processes (Zeevaart and Creelman, 1988; Schwartz *et al.*, 2001). Carotenoids with a β-ring end group are the precursors of vitamin A and therefore are fundamental for animal nutrition, including humans, which cannot synthesize this vitamin. The beneficial effects of carotenoids are also derived from their potent antioxidant activity. All these properties have provided evidence linking carotenoid intake in the human diet with protection against

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Abbreviations: β-*CHX*, β-carotene hydroxylase; HPLC-PDA, high-performance liquid chromatography-photodiode array detector; β-*LCY*, lycopene β-cyclase; ε-*LCY*, lycopene ε-cyclase; MEP, 2-C-methyl-D-erythritol 4-phosphate; *PDS*, phytoene desaturase; *PSY*, phytoene synthase; *ZDS*, ζ-carotene desaturase; *ZEP*, zeaxanthin epoxidase.

certain cancers, cardiovascular diseases, and other degenerative processes (Fraser and Bramley, 2004; Krinsky and Johnson, 2005; Rao and Rao, 2007).

Carotenoids also provide the yellow, orange or red coloration characteristic of many flowers and fruits, to attract animals for pollination or for the dispersal of seeds (Bartley and Scolnik, 1995). Citrus fruits display a wide range of colorations due to the accumulation of specific carotenoids that substantially change in the peel and pulp of the different species and varieties (Gross, 1987; Fanciullino *et al.*, 2006; Xu *et al.*, 2006; Matsumoto *et al.*, 2007). Recently, genes encoding for enzymes of the main steps of the carotenoid biosynthetic pathway have been identified and their expression studied in fruit tissues of different citrus species during natural (Kita *et al.*, 2001; Kato *et al.*, 2004; Rodrigo *et al.*, 2004; Alos *et al.*, 2006; Tao *et al.*, 2007; Alquezar *et al.*, 2008; Fanciullino *et al.*, 2008) or ethylene-induced (Rodrigo and Zacarias, 2007) fruit ripening. Figure 1 shows a schematic representation of the main steps of the carotenoid biosynthetic pathway. The peel of immature citrus fruit shows a carotenoid profile characteristic of chloroplast-containing tissue, with lutein (β,ε-xanthophyll) being the main carotenoid. At the onset of fruit maturation, the content of lutein declined in parallel with the accumulation of specific β,β-xanthophylls, as 9-*Z*-violaxanthin, which is the major carotenoid in the peel and pulp of orange-coloured mature fruit, such as oranges and mandarins. The massive increase in total carotenoids and β,β-xanthophylls occurring in the peel of orange and mandarin fruits during the transition from chloroplast to chromoplast is concomitant with the induction of phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), and β-carotene hydroxylase (*β-CHX*) gene expression (Kato *et al.*, 2004; Rodrigo *et al.*, 2004; Alquezar *et al.*, 2008). Cyclization of lycopene is a key branching point in the carotenogenesis of *Citrus* fruits, since the shift from the β,ε-branch to the β,β-branch of the pathway determinates the change in carotenoid accumulation and composition during fruit colouration. Two genes encoding lycopene cyclases (*LCY*) have been identified in citrus: *ε-LCY* and *β-LCY*, and the ability of both enzymes to cycle lycopene has been confirmed (Inoue *et al.*, 2006). β-LCY forms two β-rings at both extremes of the lineal molecule of lycopene, yielding β-carotene, while ε-LCY introduces a single ε-ring generating δ-carotene. The expression of *ε-LCY* is down-regulated during the transition from chloroplast to chromoplast, while that of *β-LCY* is constitutive or slightly increases (Kato *et al.*, 2004; Rodrigo *et al.*, 2004; Alquezar *et al.*, 2008; Fanciullino *et al.*, 2008). Accumulation of both *LCY* transcripts has been also detected in leaves (Rodrigo *et al.*, 2004).

In other carotenogenic fruits, the regulation of *LCY* genes has been shown to be critical for the specific accumulation of lycopene (Bramley, 2002). In tomato, a model fruit for the study of carotenogenesis, down-regulation of *ε-LCY* and *β-LCY* is the mechanism responsible for the massive accumulation of lycopene during fruit ripening (Pecker *et al.*, 1996; Ronen *et al.*, 1999).

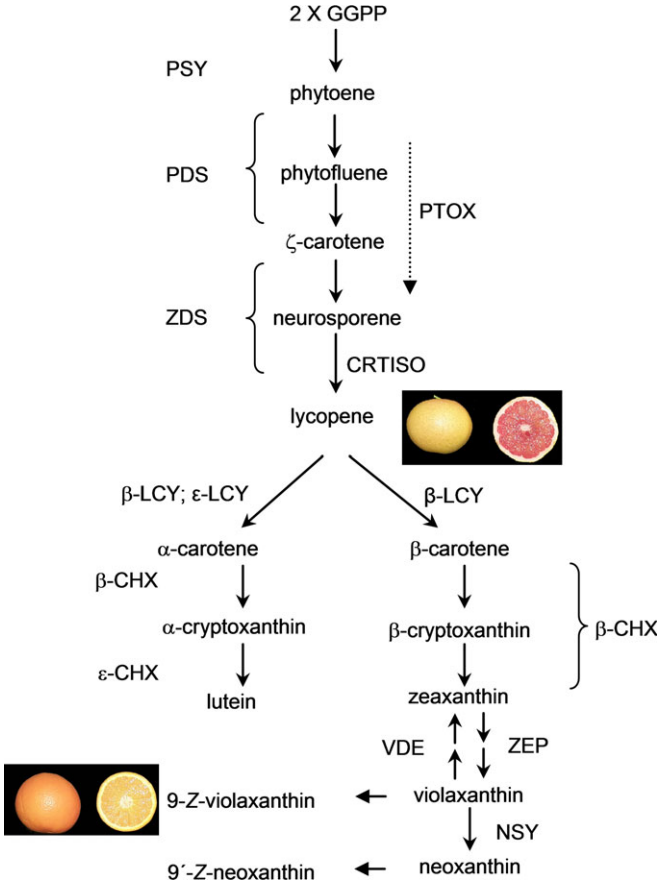

Fig. 1. Schematic diagram of the carotenoid biosynthesis pathway in plants. GGPP, geranylgeranyl diphosphate; *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, ζ-carotene desaturase; *PTOX*, plastid terminal oxidase; *CRTISO*, carotene isomerase; *ε-LCY*, lycopene ε-cyclase; β-*LCY*, lycopene β-cyclase; *β-CHX*, β-carotene hydroxylase; ε-*CHX*, ε-carotene hydroxylase; *ZEP*, zeaxanthin epoxidase; *VDE*, violaxanthin de-epoxidase; *NSY*, neoxanthin synthase. Internal and external aspect of mature Navel orange (*Citrus sinensis*) and Star Ruby grapefruit (*Citrus paradisi*) used in this study, are located in the pathway side to the major carotenoid accumulating in the pulp of full-coloured fruit.

Moreover, the existence of a chromoplast-specific lycopene β-cyclase (*CYC-B* gene) which shows a transient expression at the breaker stage, explains the *de novo* synthesis of β-carotene during tomato fruit development (Ronen *et al.*, 2000). Analysis of *Delta*, *Beta*, and *old-gold* tomato mutants has also highlighted the major role of lycopene cyclases in the tomato carotenoid complement. In the *Delta* mutant, up-regulation of *ε-LCY* during tomato fruit ripening correlates with the accumulation of δ-carotene (Ronen *et al.*, 1999). The pale orange coloration of *Beta* mutant fruits is due to an important increase in the transcription of the *CYC-B* gene which leads to a higher accumulation of β-carotene than in wild-type fruit. By contrast, the *old-gold* mutant carries a null allele of *CYC-B* resulting in an elevated concentration of lycopene and a reduction of β-carotene (Ronen *et al.*, 2000). In pepper, the function of

cyclases has been also examined. The expression of the *β-LCY* gene is low and constitutive during fruit maturation; however, the capsanthin-capsorubin synthase (*CCS*) gene, which encodes a protein which also has lycopene β-cyclase activity, is highly induced during fruit coloration. Thus, the simultaneous action of both β-LCY and CCS activities has been postulated to be responsible for the massive and specific channelling of carotenes into the β,β-branch during the ripening of red pepper (Hugueney *et al.*, 1995). Recently, a *β-LCY* gene from watermelon has been isolated and it has been proposed to be a determinant of the canary yellow and red flesh coloration (Bang *et al.*, 2007). Transcriptional profiling of the *β-LCY* gene was similar in the flesh of yellow and red watermelon, but polymorphisms were detected in the coding region of the specific sequences co-segregating with each colour phenotype. Therefore, it appears that a critical mutation in the red watermelon *β-LCY* allele might reduce β-cyclase activity, resulting in the accumulation of lycopene (Bang *et al.*, 2007).

The presence of lycopene in *Citrus* fruits is an uncommon feature. Most of the lycopene-accumulating mutants have been identified in grapefruit (*Citrus paradisi*) and pummelo (*Citrus grandis*), and only three in orange (*Citrus sinensis*): Shara, Cara Cara, and Hong Anliu (Monselise and Halevy, 1961; Lee, 2001; Liu *et al.*, 2007). Due to the high antioxidant activity and health-promoting effects of lycopene (Omoni and Aluko, 2005), and to the attractive colouration provided by this lineal carotene to citrus fruits, extensive investigations into the mechanisms involved in the accumulation of lycopene in orange mutants have recently been addressed. Biochemical data suggest that different regulatory mechanisms operate between lycopene-accumulating mutants of orange and red grapefruit (Fanciullino *et al.*, 2006; Liu *et al.*, 2007; Tao *et al.*, 2007; Alquezar *et al.*, 2008). The pulp of Cara Cara contains higher levels of linear carotenes, including lycopene, without affecting the normal complement of β,β-xanthophylls. By contrast, in red grapefruit, accumulation of lycopene is accompanied by an increase in β-carotene and a considerable reduction of β,β-xanthophylls (Xu *et al.*, 2006). Expression analysis of isoprenoid and carotenoid biosynthetic genes in fruits of the Cara Cara mutant suggest a higher induction of the expression of MEP-isoprenoid genes as the possible origin of its abnormal carotenoid profile (Alquezar *et al.*, 2008). In the orange mutant Hong Anliu, an increased expression of early carotenoid biosynthetic genes could account for the accumulation of lycopene and other upstream carotenoids (Liu *et al.*, 2007). To date, the molecular basis of lycopene accumulation in red grapefruit has not been analysed, even though alterations in β-LCY and/or β-CHX have been postulated as the possible mechanisms responsible for its particular carotenoid profile (Xu *et al.*, 2006; Fanciullino *et al.*, 2007).

The objective of this investigation was to isolate and characterize new β-LCY from citrus fruits that may explain the massive accumulation of β,β-xanthophylls occurring during the ripening of orange fruits. In the present work, a novel chromoplast-specific lycopene β-cyclase gene, so-called *β-LCY2*, which is highly induced in the peel and pulp of Navel orange fruit during ripening, is reported. Functional analysis by a colour complementation assay in *E. coli*, demonstrated that *β-LCY2* encoded a *bona fide* β-cyclase. It was then hypothesized that this new *β-LCY2* gene might be involved in the molecular mechanism underlying lycopene accumulation in the red grapefruit. To elucidate this, the expression of the chromoplast-specific *β-LCY2* gene, as well as the expression of another seven carotenoid biosynthetic genes, were analysed in the peel and pulp of Star Ruby red grapefruit, which is one of the red grapefruit with higher lycopene content, and compared with the corresponding tissues of ordinary Navel orange. The study is supplemented with functional assays of the *β*-LCY2 alleles isolated from the red grapefruit Star Ruby.

Materials and methods

Plant material and treatments

Plant material used for the experiments was collected from adult trees of orange (*Citrus sinensis* L. Osbeck cv. Washington Navel) and red grapefruits (*Citrus paradisi* cv. Star Ruby) both grafted onto Citrange carrizo rootstocks and grown in The Citrus Germplasm Bank at the Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain) and subjected to standard cultural practices. At each sampling date, at least 30 fruits were collected from three adult trees from the outer part of the canopy. Fruits were collected at different developmental stages, from immature green to full-coloured stage, during two consecutive seasons (2004/2005 and 2005/2006). Young (less than 4 months old) and mature (more than 8 months old) leaves, young stems, and petals (pre- and post-anthesis) from the same orange trees were also collected. The ethylene degreening experiment was carried out using orange fruits, harvested at the end of October (*a*/*b* ratio of -0.64 ± 0.03), which were just at the onset of natural degreening (Rodrigo *et al.*, 2004). Fruits were incubated in an ethylene-free atmosphere (control fruit) or in an atmosphere of 10 $\mu l\ l^{-1}$ ethylene in 25 l tanks at 20 °C and 85–90% RH in the dark for up to 7 d (Rodrigo and Zacarías, 2007). To avoid excess respiratory CO_2, $Ca(OH)_2$ powder was introduced in the tanks and fruit were ventilated every day. After 3 d and 7 d of incubation under the different conditions, fruit colour was measured and flavedo was excised from the whole fruit.

The external colour of the fruit was measured on three locations around the equatorial plane of the fruit using a Minolta CR-330 colorimeter. Colour is expressed as the *a*/*b* Hunter ratio (Stewart and Wheaton, 1972). The *a*/*b* ratio is negative for green fruit, the zero value corresponds to yellow fruit at the colour break, and is positive for orange-coloured fruit. For each developmental stage or treatment, at least 30 fruits were used to measure colour development and 10 fruits for each sampling time to collect fruit tissues. After colour measurement, the flavedo tissue (outer coloured part of the fruit) was removed by a scalpel and all the plant material was frozen

in liquid nitrogen, ground to a fine powder. and stored at –80 °C until analysis.

Isolation of lycopene β*-cyclases from orange (*Citrus sinensis *cv. Navel) and red grapefruit (*Citrus paradisi *cv. Star Ruby), sequence analysis and functional expression in* Escherichia coli

The *β-LCY1* and *β-LCY2* cDNAs containing the complete coding sequence from *C. sinensis* (cv. Navel) and *C. paradisi* (cv. Star Ruby) were obtained using a RT-PCR approach. Synthesis of cDNAs was performed with 1 μg of total RNA from flavedo and pulp of fruits. The reaction was carried out in the presence of 500 ng of oligo-dT and 200 units of SuperScript II Reverse transcriptase (Gibco BRL, Germany). For PCR amplification of *β-LCY1*, primers MJ56 and MJ57 were designed on the sequence of the lycopene β-cyclase from *C. sinensis* (GenBank accession number AY094582) available in public sequence databases. For the isolation of *β-LCY2*, primers MJ69 and MJ66 were based on the sequence of a putative capsanthin capsorubin synthase homologue from *C. sinensis* (GenBank accession number AF169241). Sequences of the primers used are shown in Table 1. Cycling parameters for RT-PCR were: 94 °C for 3 min, 30 cycles of 94/58/72 °C for 30/30/90 s, respectively, and 72 °C for 10 min. Purified PCR products were cloned into pGEM-T Easy vector (Stratagene). Clones with the gene sequence in the sense orientation with respect to the *lacZ* promoter were selected. The identity of gene sequences and orientation in the plasmid were verified by sequencing. The recombinant plasmids harbouring the *β-LCY1* and *β-LCY2* genes from *C. sinensis* were designated pGEM-CsβLCY1 and pGEM-CsβLCY2, respectively, and from *C. paradisi* pGEM-CpβLCY1 and pGEM-CpβLCY2, respectively.

Prediction of transit peptide of Csβ-LCY1 and Csβ-LCY2 proteins was carried out using the ChloroP 1.1 Prediction Server program (Emanuelsson *et al.*, 1999). Sequences encoding plant β-LCYs were recovered by homology search in sequence databanks using the program BLAST (Altschul *et al.*, 1990) at the NCBI (Bethesda, USA) and only full-length amino acid sequences were used for phylogenetic analysis. A phylogenetic tree was generated using the Neighbor–Joining method (Saitou and Nei, 1987) included in the ClustalW program (Thompson *et al.*, 1994) and bootstrap re-sampling analysis (1000 replicates) was performed.

Functional assays were carried out in *E. coli* XL1-Blue strain. *E. coli* cells were transformed with plasmid pACCRT-EIB (a gift from Professor Misawa, Marine Biotechnology Institute, Iwate, Japan), which harbours the *Erwinia uredovora* genes necessary for lycopene production in *E. coli* (Misawa and Shimada, 1998), and used as host cells for pGEM-CsβLCY1, pGEM-CsβLCY2, pGEM-CpβLCY1 and pGEM-CpβLCY2 or empty pGEM-T plasmid as control. The double-transformants were plated in LB supplemented with ampicillin (100 μg ml^{-1}) and chloramphenicol (50 μg ml^{-1}) and incubated for 48 h at 30 °C. To standardize culture conditions, a culture of 5 ml of LB plus antibiotics was prepared by colony inoculation with double-transformants and incubated for 12 h at 37 °C. Then, a 10 μl aliquot was used to inoculate 20 ml of LB medium supplemented with the selective antibiotics and incubated for 48 h at 30 °C in the dark to maximize carotenoid production. All assays were done with at least two independent clones and cultures were prepared in triplicate.

Table 1. Primers used in the amplification of full-length sequences of *β-LCY1* and *β-LCY2* genes and for expression analysis of *β-LCY1*, *β-LCY2*, and *β-CHX* genes

In the primer sequences for the amplification of full-length sequences of *β-LCY1* and *β-LCY2* genes, start and stop codons of the predicted proteins were included and are underlined.

Gene	Primer	Primer sequence (5′→3′)	Orientation[a]
β-LCY1	MJ56	GCTCTAGCCTTGTAGGAAAGCCATGG	S
	MJ57	GCGAATTCCGTGTGCACCTTAATCTGTATC	AS
	MJ136	GAACCAGGAGCTTAGGTCTG	S
	MJ137	GCTAGGTCTACAACAAGGCC	AS
β-LCY2	MJ35	ACTCTAGACCTATTTCCATTAGGCCGC	S
	MJ36	GCCTCGAGCCTTGACACTATGACGCG	AS
	MJ66	GCCTCGAGATCTTCAAATGGTTTCAAG	AS
	MJ69	GCATGGCAACTCTTCTTAGCCCG	S
	MJ67	CTCATCGCGTCATAGTGTCAAGG	S
	MJ68	AGCTCGCAAGTAAGGCTCATTCCC	AS
	MJ130	CCCTATTTCCATTAGGCCGC	S
	MJ138	CACGTCATATCGAATACGATC	AS
β-CHX	MJ126	GGCTCATAAAGCTCTGTGGC	S
	MJ127	CCAGCACCAAAACAGAGACC	AS

[a] S, Sense; AS, antisense.

HPLC analysis of carotenoids

Carotenoids from *E. coli* cells were extracted from 15 ml of cultures. Cultures were centrifuged at 4000 *g* for 5 min and the bacterial pellet was washed twice with water. The pellet was then resuspended in 1 ml of acetone, mixed vigorously for 30 s and cells centrifuged for 2 min at 13 000 *g*. The coloured supernatant was centrifuged again for 2 min at 13 000 *g*, placed in a clean tube, dried with nitrogen, and stored at –20 °C until HPLC analysis.

Carotenoids from flavedo and pulp of *Citrus* fruits were extracted as previously described by Rodrigo *et al.* (2003) with slight modifications. Briefly, freeze-ground material of flavedo (500 mg) or pulp (2 g) was extracted with a mixture of methanol and 50 mM TRIS-HCl buffer (pH 7.5) containing 1 M NaCl and partitioned against chloroform until all the colour was removed from the plant material. Pooled organic phases were dried under vacuum and saponified overnight using a KOH methanolic solution. The carotenoids were subsequently re-extracted with diethyl ether. The extracts were reduced to dryness by rotary evaporation and keep under a nitrogen atmosphere at –20 °C until HPLC analysis. Carotenoid extracts were prepared

for HPLC analysis by dissolving in chloroform:MeOH:acetone (5:3:2 by vol.). Chromatography was carried out with a Waters liquid chromatography system equipped with a 600E pump and 996 photodiode array detector, and data analysed with Empower software (Waters). Carotenoid pigments were separated by HPLC using a C_{30} carotenoid column (250×4.6 mm, 5 μm) coupled to a C_{30} guard column (20×4.0 mm, 5 μm) (YMC Europe GMBH, Germany) with ternary gradient elution of MeOH, water, and methyl *tert*-butyl ether (MTBE) (Alquezar *et al.*, 2008). Carotenoids were identified by their retention time, absorption, and fine spectra (Rouseff *et al.*, 1996; Britton, 1998). For each elution, a Maxplot chromatogram was obtained which plots each carotenoid peak at its corresponding maximum absorbance wavelength. The carotenoid peaks were integrated at their individual maximal wavelength and their content was calculated using calibration curves of zeaxanthin (Sigma), β-carotene (Sigma), lycopene (Sigma), β-cryptoxanthin (Extrasynthese), lutein (Sigma) for lutein, violaxanthin, and neoxanthin isomers and β-apo-8′-carotenal (a gift from Hoffman-LaRoche) for β-citraurin. Phytoene and phytofluene were previously purified as is described in Pascual *et al.* (1993) by thin layer chromatography from carotenoid extracts of Pinalate orange fruit, a mutant which accumulates substantial amounts of these carotenes (Rodrigo *et al.*, 2003).

Samples were extracted at least twice and each analytical determination was replicated. All operations were carried out on ice under dim light to prevent photodegradation, isomerization and structural changes of carotenoids.

Northern and Southern blot hybridization and probe labelling

Total RNA was isolated from plant material as previously described (Rodrigo *et al.*, 2004). Northern blot analysis was carried by electrophoresis of denatured total RNA (10 μg) in 1% (w/v) agarose-formaldehyde/MOPS (3-[*N*-morpholino]-propanesulphonic acid) gel and blotted onto nylon membranes (Hybond-N, Amersham-Bioscienc) essentially as described by Sambrook *et al.* (1989).

Southern DNA was extracted from young leaves as described by Taylor *et al.* (1993). Samples of genomic DNA (10 μg) were digested with selected restriction enzymes, electrophoresed on 1% (w/v) agarose gel and transferred as above.

Probes were derived from cDNA clones of the carotenoid biosynthetic genes *PSY, PDS, ZDS, β-LCY, ε-LCY, β-CHX, ZEP* (Rodrigo *et al.*, 2004) and 26rDNA (Ballester *et al.*, 2006) from *C. sinensis*. For *β-LCY2* transcript detection, a fragment of 250 bp was amplified with the specific-primers MJ35 and MJ36 (Table 1) from the plasmid pGEM-Csβ-LCY2. All fragments were labelled with [α-^{32}P]dATP by linear PCR amplification with the corresponding antisense primers using the Strip-EZ PCR Kit (Ambion, Huntingdon, UK) following the manufacturer's instructions. An equivalent number of counts (10^6 cpm ml^{-1}) of each probe were used for hybridization. Northern blots were exposed to Phosphorscreens and the images read on a FLA-3000 laser scanner (Fujifilm, Tokyo, Japan). In order to determine relative gene expression, the signal in each band was determined using ImageGauge 4.0 (Fujifilm) software. Filters were stripped off following the instructions in the Strip-EZ PCR kit and re-hybridized several times. Finally, filters were hybridized to the 26S rDNA *C. sinensis* probe to normalize the hybridization of each gene by calculating the ratio between the hybridization signal of each mRNA and that obtained using the 26S rDNA *C. sinensis* probe. For each gene a value of 100 was assigned to the normalized signal of orange Navel flavedo at full-coloured stage and expression level of the rest of the samples referred to it.

Quantitative real-time PCR analysis

Total RNA was treated with DNase (Ambion, Huntingdon, UK) and accurately quantified by fluorometric assay with the RiboGreen dye (Molecular Probes) following the manufacturer's instructions, in order to normalize mRNA levels as described by Alos *et al.* (2006). Quantitative real-time PCR (RT-PCR) was performed with a LightCycler 2.0 Instrument (Roche) and fluorescence was analysed using LightCycler Software version 4.0. One-step RT-PCR was carried out on 100 ng total RNA adding 2.5 units of MultiScribe Reverse Transcriptase (Applied Biosystems), 1 unit RNase Inhibitor (Applied Biosystems), 2 μl LC FastStart DNA MasterPLUS SYBR Green I (Roche), and gene specific primers in a total volume of 10 μl. Primers pairs for *β-LCY1* (MJ136 and MJ137, 0.1 μM), *β-LCY2* (MJ130 and MJ138, 0.1 μM), and *β-CHX* (MJ126 and MJ127, 0.3 μM), detailed in Table 1, were designed based on *Citrus* coding sequences isolated from fruit and available in databases (GenBank accession numbers AY094582, AF169241, and DQ228870, respectively). The RT-PCR procedure consisted of 48 °C for 30 min, 95 °C for 10 min followed by 35 cycles at 95 °C for 10 s, 5 s of melting at specific temperature (60 °C, 58 °C, and 61 °C, for *β-LCY1*, *β-LCY2*, and *β-CHX*, respectively) and 72 °C for 10 s. Fluorescence intensity data were acquired during the 72 °C extension step and specificity of the reactions was checked by post-amplification dissociation curves. To transform fluorescence intensity measurements into relative mRNA levels, a 10-fold dilution series of a RNA sample was used as a standard curve. Values were the mean of at least three independent analyses. An expression value of 100 was arbitrarily assigned to the orange Navel flavedo at the full-coloured stage and the rest of the values referred to it.

Results

Isolation and expression analysis of a novel chromoplast-specific lycopene β-cyclase from Citrus sinensis

A search through public sequence databases revealed a sequence from *C. sinensis* (GenBank accession number

AAF18389) that shared a 55% of identity at the level of amino acid with the Csβ-LCY1 sequence and between 69–75% to CYC-B, a β-LCY responsible for the β-ring formation in tomato chromoplasts (Ronen *et al.*, 2000), and to the capsanthin capsorubin synthase (CCS) from pepper, an enzyme with a reaction mechanism similar to that of LCY (Bouvier *et al.*, 1997; al Babili *et al.*, 2000). Since neither capsanthin nor capsorubin are present in the flavedo and pulp from *C. sinensis*, it was envisaged that this gene could encode for a novel β-LCY. Therefore, this gene was tentatively named as *Csβ-LCY2*. To clone *Csβ-LCY1* and *Csβ-LCY2* from orange fruits, a RT-PCR based strategy was adopted using as templates cDNAs from flavedo and pulp from coloured mature orange fruits. After gene sequencing, most of the variability found between the polypeptides Csβ-LCY1 and Csβ-LCY2 was detected in the N-terminal region (Fig. 2A) in which transit peptides of similar length were predicted. Moreover, Csβ-LCY2 contains partial and full conserved motifs previously described as characteristics of plant lycopene cyclase such as: plant β-LCY conserved regions, a dinucleotide-binding domain, cyclase motifs I and II, which are essentials domains for catalytic activity, and a charged region (Hugueney *et al.*, 1995; Cunningham *et al.*, 1996; Bouvier *et al.*, 1997; Fig. 2A). The relationship between Csβ-LCY1, Csβ-LCY2, and the other 11 plant β-LCYs was investigated by generating a phylogenetic tree (Fig. 2B). The neoxanthin synthase (NSY) sequences from tomato and potato, and pepper CCS were also included in the analysis since these enzymes and β-LCY catalyse analogous reactions (Bouvier *et al.*, 1997, 2000) and a common origin for all of them has been proposed (al Babili *et al.*, 2000; Krubasik and Sandmann, 2000). The phylogenetic tree showed that the two *Citrus* β-LCYs map in different subfamilies (Fig. 2B). Csβ-LCY1 is grouped with the plant β-LCYs cluster, whose sequences showed an overall identity of 78–87% (86–93% similarity), while Csβ-LCY2 is more closely related to *Solanum* NSYs, and tomato CYC-B and pepper CCS, which also have β-LCY activity (Hugueney *et al.*, 1995; Ronen *et al.*, 2000). Sequence comparison between full-length cDNAs and genomic sequences of *Citrus β-LCY1* and *β-LCY2* revealed an intronless structure for both genes. DNA blot hybridization with total genomic DNA indicated that both *β-LCY1* and *β-LCY2* probably exist in a single copy in the *C. sinensis* genome (data not shown).

Expression of *Csβ-LCY2* in different orange tissues was investigated and compared with that of *Csβ-LCY1*. Transcript corresponding to *Csβ-LCY1* was detected in leaves, roots, petals, and fruit tissues while that of *Csβ-LCY2* was not or hardly detectable in green tissues (Fig. 3). During

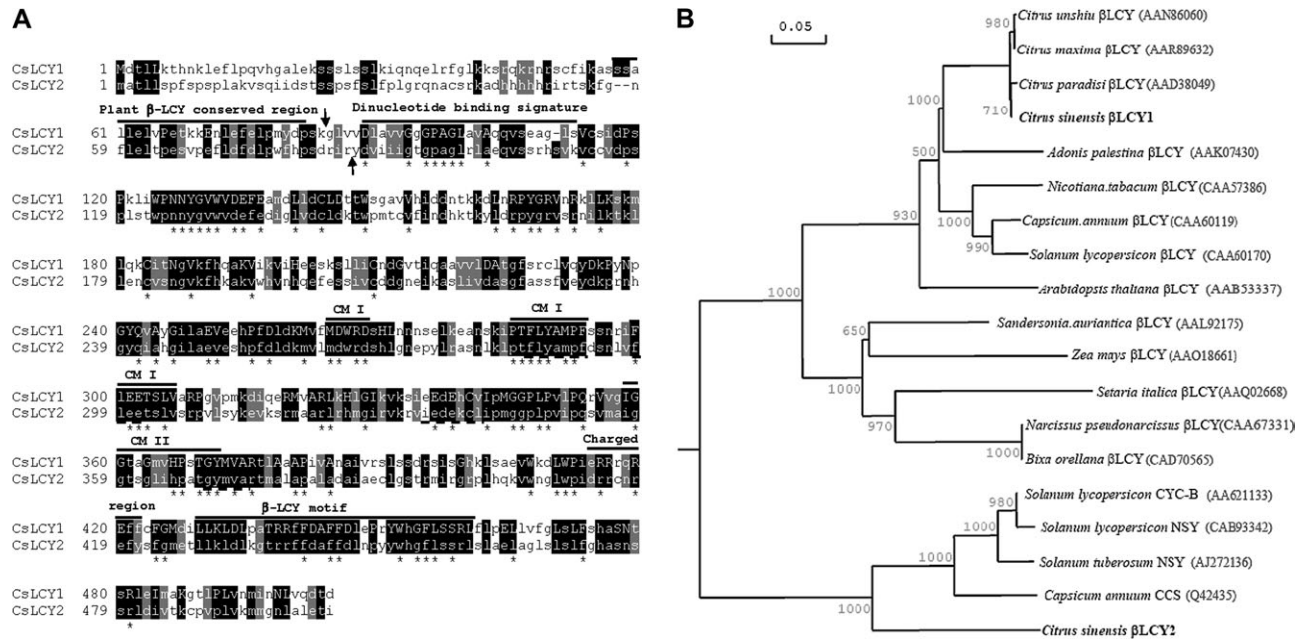

Fig. 2. (A) Alignment of deduced amino acid sequences of *Csβ-LCY1* and *Csβ-LCY2*. The alignment was created by using ClustalW program. Numbers on the left denote the number of amino acid residues. Residues identical for both sequences in a given position are in white text on a black background, those identical in all plant β-LCYs (including tomato CYC-B and pepper CCS) are in capital letters on the Csβ-LCY1 sequence, while those also conserved in ε-LCYs are marked with an asterisk (*). The most likely points for chloroplast precursor cleavage are indicated with arrows. Characteristic regions of plant LCYs are indicated on the Csβ-LCY1 sequence as plant β-LCY conserved region, di-nucleotide binding signature, cyclase motifs (CM) I and II, charged region, and β-LCY motif (Hugueney *et al.* 1995; Cunningham *et al.* 1996). Domains described as essential for β-LCY activity are underlined (Bouvier *et al.* 1997). (B) Phylogenetic tree generated based on alignment of deduced amino acid sequences of plant β-LCYs, NSYs, and CCS. The tree was constructed on the basis of the Neighbor–Joining method (Saitou and Nei, 1987). The bootstrap values on the nodes indicate the number of times that each group occurred with 1000 replicates. Only bootstrap values greater than 500 are shown. Accession numbers of protein sequences are in parenthesis.

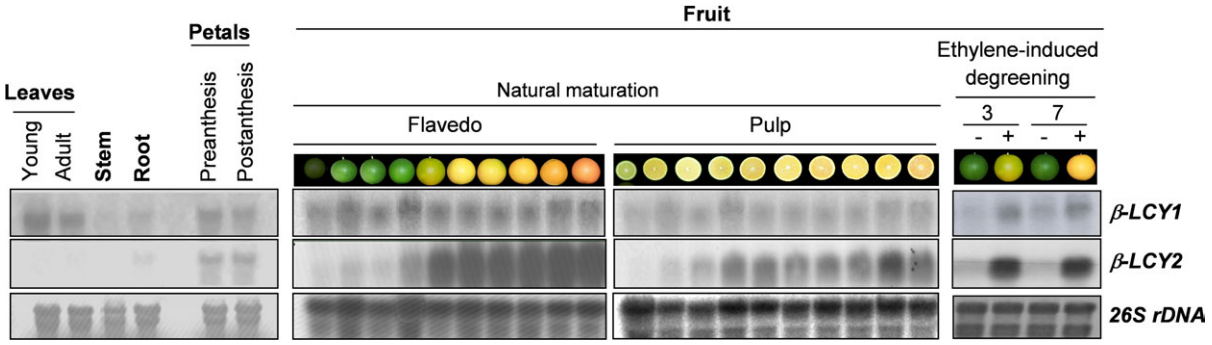

Fig. 3. Accumulation of *Csβ-LCY1* and *Csβ-LCY2* transcripts in different orange (*Citrus sinensis* cv. Navel) tissues. In the degreening experiment total RNA from flavedo of control fruits (–) or ethylene treated (+) (10 μl l^{-1}) after 3 d and 7 d of treatment was used for expression analysis. Each lane was loaded with 10 μg of total RNA. The RNA was fractionated on a 1% agarose-formaldehyde gel, blotted onto nylon membrane, and hybridized with the correspondent probe. Note that for thr *Csβ-LCY1* probe, the exposure time was between 4–15 times higher than for the *Csβ-LCY2* probe. Membrane staining with methylene blue shows the rRNA bands. Expression data are representative of at least two independent experiments.

fruit ripening, the expression of *Csβ-LCY2* was strongly induced in both flavedo and pulp, in contrast to *Csβ-LCY1* whose expression was almost invariable during the process (Fig. 3). The expression levels of both β-cyclase genes were higher in the peel than in the pulp. Application of ethylene clearly stimulated the expression of *Csβ-LCY2* (Fig. 3), as occurs with other ripening up-regulated carotenogenic genes in citrus fruits (Rodrigo and Zacarias, 2007), and accumulation of the mRNA was also higher than that of *Csβ-LCY1*.

Functional characterization of Citrus sinensis β-LCY2 *in* Escherichia coli

Lycopene-accumulating *E. coli* cells harbouring a lycopene biosynthetic plasmid (pACCRT-EIB; Misawa and Shimada, 1998) were cotransformed with plasmids pGEM-CsβLCY1, pGEM-CsβLCY2 or pGEMT without insert as the negative control. Carotenoids were extracted from bacteria and analysed by HPLC (Fig. 4). The HPLC elution profiles from control cultures harbouring the empty cloning vector displayed predominantly a single peak, whose retention time and absorbance spectrum corresponded to lycopene (Fig. 4A, peak 1). Chromatograms obtained from extracts of the lycopene-accumulating strain cotransformed with the pGEM-CsβLCY1 plasmid showed a new peak with a retention time and spectrum characteristics corresponding to β-carotene (Fig. 4B, peak 2), whereas that of lycopene was virtually undetectable. This result indicates that the protein CsβLCY1 generated sufficient enzyme activity to convert almost all the lycopene produced by the cells to β-carotene. In the extracts of the lycopene-accumulating bacteria cotransformed with the plasmid pGEM-CsβLCY2, both lycopene (peak 1) and β-carotene (peak 2) were detected (Fig. 4C). It is interesting to mention that the monocyclic δ-carotene, which results from the cyclization of one end of the lycopene molecule, was not detected in any of the assays performed. The β-LCY activity of these experiments was calculated for each construct as the percentage of lycopene converted into β-carotene in at least eight independent assays. The resulting activities were 95.45±1.20 for Csβ-LCY1 and of 38.33±11.40 for Csβ-LCY2, demonstrating that both genes encoded functional lycopene β-cyclases.

*Comparative analysis of carotenoid biosynthetic gene expression in orange (*C. sinensis *cv. Navel) and red grapefruit (*C. paradisi *cv. Star Ruby) during fruit ripening*

In order to understand the molecular basis of lycopene accumulation in red grapefruit, a comparative analysis of the expression of carotenoid biosynthetic genes in flavedo and pulp during fruit ripening was carried out in Star Ruby which is one of the most red-coloured grapefruits (Rouseff *et al.*, 1992) and in Navel orange, a standard orange-pigmented fruit. The red colour in grapefruit is due to the accumulation of lycopene which represents nearly 50% of total carotenoid content in the pulp (Table 2). In mature Navel fruit, the characteristic orange colour is determined by the accumulation of β,β-xanthophylls, mainly 9-*Z*-violaxanthin, which represented more than 60% and 90% of total carotenoids in the peel and pulp, respectively (Table 2). Besides the presence of lycopene, the high concentration of other carotenes in Star Ruby tissues, such as phytoene, which account for 26% and 74% of total carotenoids in the pulp and in the peel, respectively (Table 2) is also noteworthy. Other interesting differences between mature fruits of both genotypes are the reduced carotenoid content in the peel (7-times lower) but increased in the pulp (2.5-times higher) in grapefruit with respect to oranges. In red grapefruit, the total carotenoid content was very similar in flavedo and pulp, an unusual feature in carotenogenic fruits (Table 2).

The expression level of eight relevant carotenogenic genes in Navel orange and Star Ruby grapefruit fruits at four developmental/ripening stages was evaluated (Fig. 5). The selected genes are involved in carotene biosynthesis (*PSY*, *PDS*, *ZDS*), lycopene *β*-cyclization (*β-LCY1* and the above characterized *β-LCY2*) and ε-cyclization (*ε-LCY*), and xanthophyll biosynthesis (*β-CHX* and *ZEP*). In general,

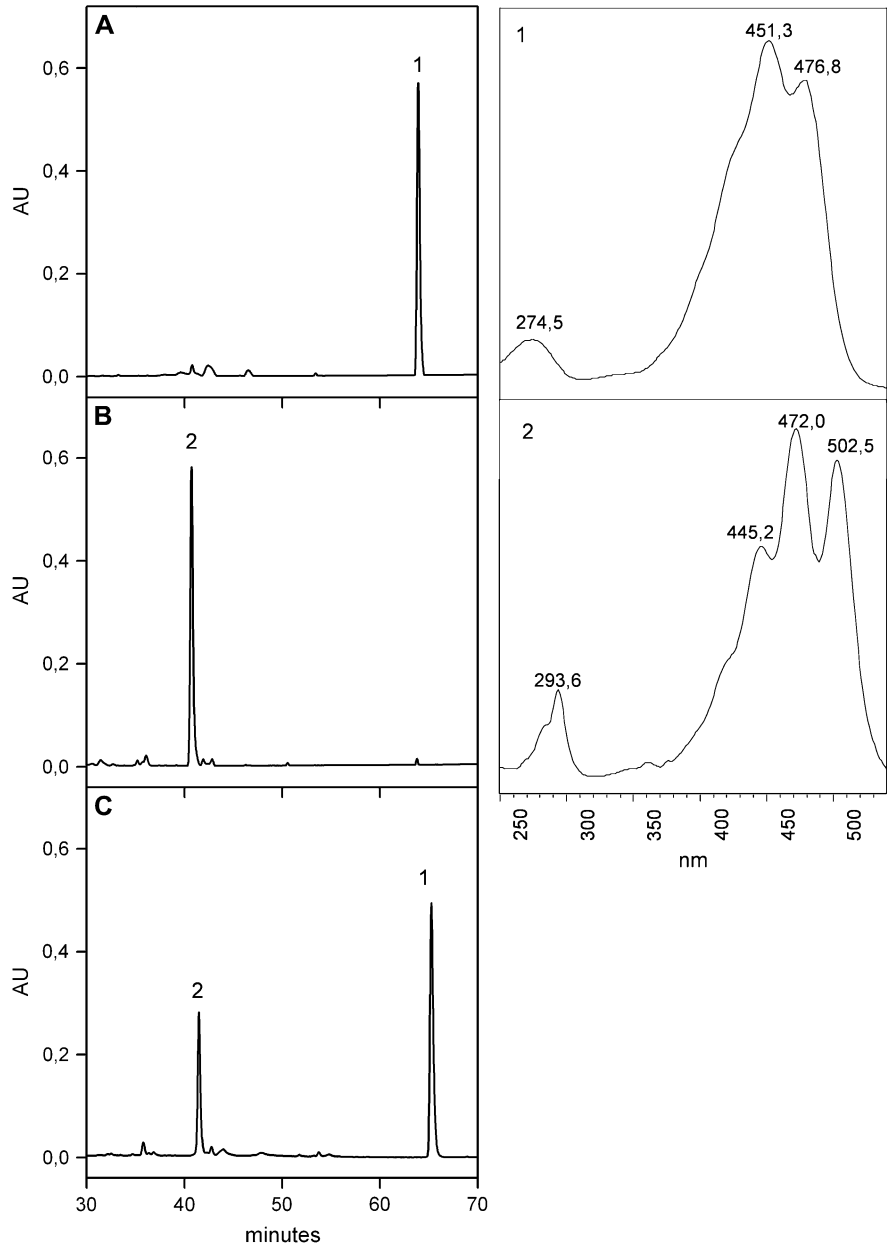

Fig. 4. Analysis by HPLC-PDA of carotenoids in *E. coli* cells that accumulate lycopene and express β-LCY1 or β-LCY2 from *Citrus sinensis*. Carotenoids were extracted from suspension cultures of cells with plasmids pACCRT-EIB and pGEM (empty vector) (A), plasmids pACCRT-EIB and pGEM-Csβ-LCY1 (B), or plasmids pACCRT-EIB and pGEM-Csβ-LCY2 (C). Absorbance spectra of the peaks are showed in boxes: peak 1, lycopene; peak 2, β-carotene.

genes of the early steps of the pathway (*PDS*, *ZDS*), *β-LCY1*, and ε-*LCY* showed a similar expression profile in Navel and Star Ruby fruit tissues, while differences were detected for *PSY*, *β-LCY2*, *β-CHX*, and *ZEP* genes (Fig. 5). The expression of *β-LCY2*, *β-CHX*, and *ZEP* increased during ripening in the flavedo of both phenotypes, but to a lower extent in Star Ruby. In the pulp, transcripts of *PSY* and *ZEP* showed a similar accumulation profile in Navel and Star Ruby but again accumulated to lower levels. Interestingly, differences in the accumulation of the mRNA corresponding to *β-LCY2* and *β-CHX* were much more remarkable in pulp tissue. The expression of *β-LCY2* and *β-CHX* clearly increased in the pulp of Navel fruit during ripening, but in Star Ruby the expression of both genes was much lower and remained nearly constant during the process (Fig. 5).

Due to the relevance of *β-LCY1*, *β-LCY2*, and *β-CHX* genes in the regulation of lycopene accumulation, and to corroborate previous results, quantitative real-time PCR analysis was performed for these genes in Navel and Star Ruby fruit tissue comparing physiological stages equivalents to those used for Northern analysis (Fig. 6). No important differences were observed for the expression of *β-LCY1*. The accumulation of *β-LCY2* and *β-CHX* transcripts was delayed and reduced in Star Ruby, and this situation was much more pronounced in the pulp than in

Table 2. Content of carotenoids in flavedo and pulp of full-coloured fruits of Navel orange (*Citrus sinensis*) and Star Ruby red grapefruit (*Citrus paradisi*)

Values are mean ±SD of at least three independent measurements, and are given in μg g^{-1} fresh weight (FW)

Carotenoid (μg g^{-1} FW)	Tissue			
	Flavedo		Pulp	
	Navel	Star Ruby	Navel	Star Ruby
Phytoene	36.56±9.91	11.15±0.89	0.39±0.09	3.36±082
Phytofluene	4.74±1.42	1.13±0.12		0.88±0.19
Lycopene		0.32±0.10		6.10±0.86
β-Carotene		0.21±0.02		2.43±0.37
β-Cryptoxanthin	1.86±0.13		0.29±0.05	
Antheraxanthin			0.80±0.12	
Zeaxanthin			0.05±0.01	
9-*Z*-Violaxanthin	46.30±2.25	1.11±0.89	3.05±0.71	
All-*E*-violaxanthin	16.07±2.10	1.06±0.03		
β-citraurin	2.80±0.09	0.05±0.01		
Total carotenes	41.10±11.33	12.82±1.15	0.39±0.09	12.90±2.36
Total xanthophylls	67.04±4.51	2.18±0.22	4.76±0.11	
Total carotenoids	108.48±15.80	15.05±1.15	5.17±0.05	12.90±2.36

the flavedo. For instance, in full-coloured fruit, the expression of *β-LCY2* and*β-CHX* in the pulp of Star Ruby was 56% and 77%, respectively, lower than in Navel (Fig. 6).

*Isolation and functional analysis of lycopene β-cyclases from red grapefruit (*C. paradisi *cv. Star Ruby)*

To evaluate whether the functionality of lycopene β-cyclases from Star Ruby grapefruit might also be altered, full-length cDNAs of *β-LCY1* and *β-LCY2* from this specie were isolated. The nucleotide sequence of *β-LCY1* from Star Ruby was identical to that previously isolated from Navel orange (*Csβ-LCY1*). Analysis of Star Ruby lycopene β-cyclase activity by colour complementation in a lycopene-accumulating strain of *E. coli*, revealed no significant differences with that of Navel oranges (83.95±10.42% of lycopene was converted into β-carotene, n=12). The nucleotide sequence of *β-LCY2* isolated from Star Ruby was 98% identical to that from Navel orange. Sequence comparison of *β-LCY2* from Navel orange and Star Ruby grapefruit revealed 27 changes in nucleotides resulting in 16 amino acid changes (Table 3). Most of the amino acid changes were conservative or located in low conserved regions over other plant β-LCYs, however, some interesting substitutions were identified. For example, changes in amino acid positions 67 and 72 (Table 3) affected the di-nucleotide binding signature, a very well conserved region in plant β-LCYs. The alteration in amino acid position 359 which implies a change from Gly to Ser is also noteworthy. This Gly residue is absolutely conserved in all plant β-LCYs, even in the CYC-B from tomato and CCS from pepper. Functional assays of the β-LCY2 from Star Ruby grapefruit revealed that the lycopene β-cyclase activity of this protein was almost null (3.27±3.19% of lycopene was converted into β-carotene, n=15).

Analysis of β-LCY2 *alleles from* Citrus *sp.*

The preceding results suggest that, in the genus *Citrus*, there may exist at least two different alleles of *β-LCY2*: one referred as *β-LCY2a* (GenBank accession number FJ516403), corresponding to the sequence originally isolated from Navel orange (*C. sinensis*) encoding a functional lycopene β-cyclase, and a second, named as *β-LCY2b* (GenBank accession number FJ516404), isolated from Star Ruby grapefruit (*C. paradisi)* which encodes for a protein with almost null activity showing chromatograms similar to that obtained with the pGEM-T empty plasmid. Sequence analysis of genomic DNA from Navel orange and Star Ruby grapefruit revealed the presence of both alleles in both genotypes. Since in previous experiments only the functional allele (*β-LCY2a*) was isolated from orange and the non-functional *(β-LCY2b*) from red grapefruit, it was hypothesized that both alleles might be differentially transcribed in Navel oranges and Star Ruby grapefruits. To test this possibility, cDNA was prepared and cloned from pulp of Navel and Star Ruby fruits at breaker and full-coloured stage. Fifty independent clones were sequenced from each genotype and, interestingly, 63% of the clones from Navel corresponded to the allele *β-LCY2a* which has β-cyclase activity, whereas in Star Ruby 74% corresponded to *β-LCY2b.* Moreover, to expand the search of the relative frequency of transcription of each *β-LCY2* allele in different *Citrus* species, an *in silico* analysis of both alleles was performed in two of the major EST Citrus databases, Citrus Functional Genomics Project (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/) and HarvEST:Citrus (http://harvest.ucr.edu/), corresponding to 315 000 high quality ESTs from 182 libraries. A total of 20 ESTs of *β-LCY2* were identified in 12 libraries and, interestingly, all were generated from fruit or flower tissues of *C. sinensis*, *C. paradisi*, *C. clementina*, and *C. reticulata* (see Supplementary Table S1 at *JXB* online). Ten sequences of *β-LCY2* were found in libraries of orange tissues and the functional allele *β-LCY2a* was the more abundant (60%), in a proportion similar to that obtained in the sequencing of cDNAs. In libraries from *Citrus reticulata*/*clementina* tissues, the proportion of functional/non-functional allele was 6:2. Only two *β-LCY2* ESTs were identified in grapefruit libraries and both corresponded to the non-functional allele.

Discussion

Cyclization of lycopene by lycopene cyclases (ε- and β-LCY) to produce α- and β-carotene is a key regulatory branching point in the carotenoid biosynthetic pathway and alterations in their regulation or enzyme activity profoundly affect carotenoid composition (Hirchsberg, 2001; Cunningham, 2002; Bramley, 2002; Botella-Pavia and Rodriguez-Concepcion, 2006; Howitt and Pogson, 2006). During

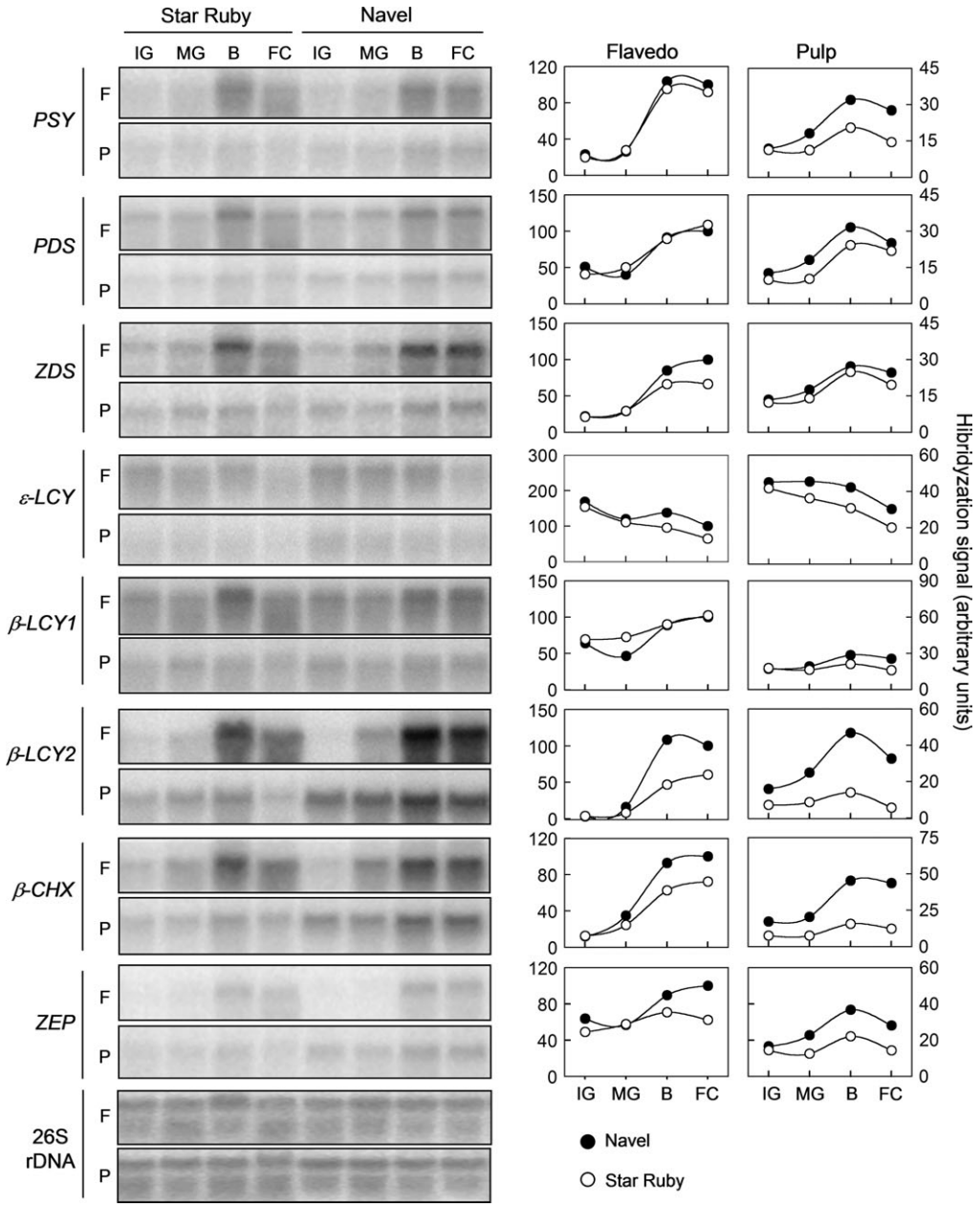

Fig. 5. Accumulation of mRNAs from carotenoid biosynthetic genes in the flavedo and the pulp of Navel orange (*C. sinensis*) (black symbols) and Star Ruby grapefruit (*C. paradisi*) (white symbols) at the IG (immature green), MG (mature green), B (breaker), and FC (full-colour) stages. All transcripts values for individual genes were normalized with respect to the corresponding value of the 26s rDNA signal. Normalized values of mRNAs accumulation in arbitrary units are represented using the FC flavedo of Navel as a reference (100).

ripening of sweet orange fruit (*C. sinensis*) a substantial accumulation of specific β,β-xanthophylls occurs in both flavedo and pulp (Gross, 1987; Kato *et al.*, 2004; Rodrigo *et al.*, 2004). This process implies an important stimulation of the β,β-cyclization of lycopene. However, the previously characterized *β-LCY* from *C. sinensis* (*Csβ-LCY1*), showed a low and relatively constant expression during fruit ripening (Kato *et al.*, 2004; Rodrigo *et al.*, 2004; Alquezar *et al.*, 2008). In this work, the isolation and functional characterization of a gene encoding a β-LCY, namely *Csβ-LCY2,* from orange fruit is reported. Taken together, results indicate that, firstly, this gene plays a major function in the carotenogenesis of citrus fruit and in the massive and specific channelling of carotenes into the β,β-branch during ripening of orange fruit and, secondly, alterations in the expression of this gene and in the function of the corresponding protein are likely to be involved in the accumulation of lycopene characteristic of red grapefruit.

The expression of *β-LCY2* was restricted to chromoplastic tissues and the up-regulation of *Csβ-LCY2* gene parallels the massive accumulation of β,β-xanthophylls accompanying orange fruit maturation (Kato *et al.*, 2004; Rodrigo *et al.*, 2004; Alquezar *et al.*, 2008; Fig. 3). Moreover, treatment of mature-green fruits with exogenous ethylene which promotes accumulation of β,β-xanthophylls (Rodrigo and Zacarias, 2007), also induced the

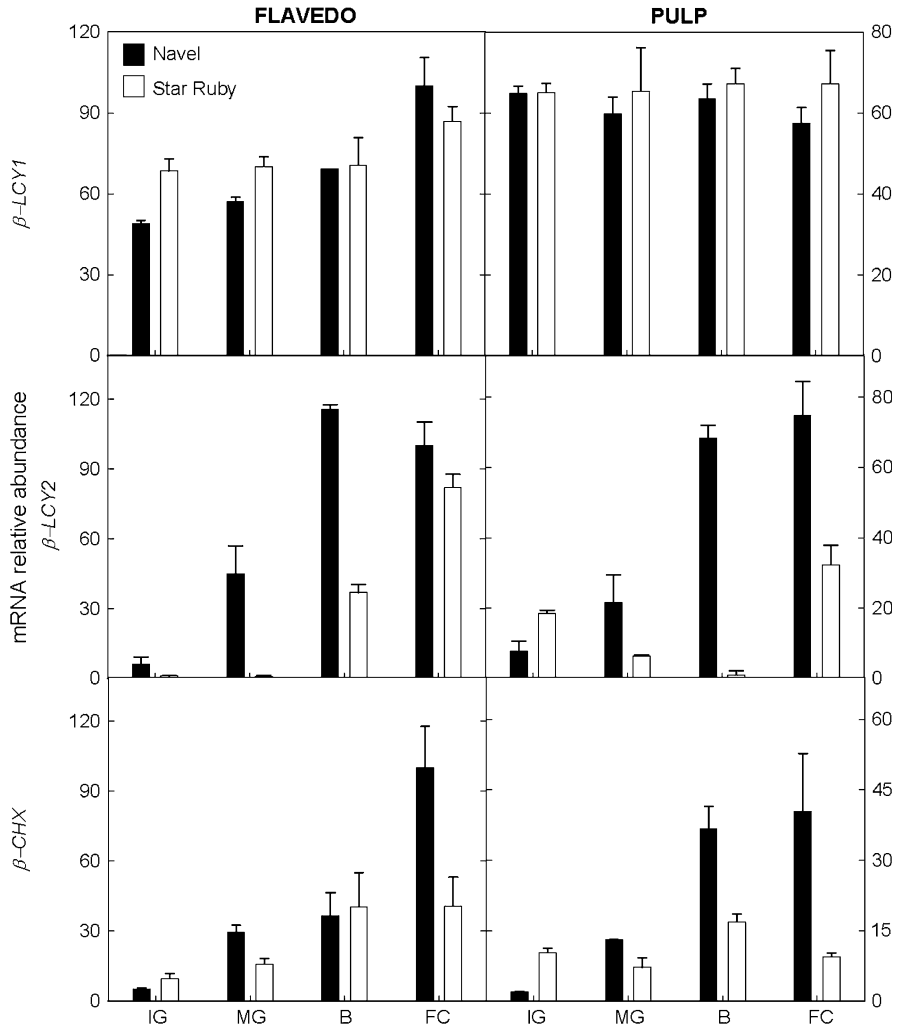

Fig. 6. Quantitative RT-PCR analysis of the expression of *β-LCY1*, *β-LCY2*, and *β-CHX* genes in the flavedo and the pulp of Navel orange (*C. sinensis*) (black bars) and Star Ruby grapefruit (*C. paradisi*) (white bars) at the IG (immature green), MG (mature green), B (breaker), and FC (full-colour) stages. The levels of expression were normalized to the amount of RNA and the value of Navel flavedo at the FC stage was set to 100. The data are means ±SD of three experimental replicates.

accumulation of *Csβ-LCY2* transcript (Fig. 3). In contrast to these patterns of expression, the transcript of the canonical *β-LCY* gene, renamed as *Csβ-LCY1*, was detected at a similar level throughout the whole maturation/ripening period in orange fruits and also in vegetative tissues (Figs 3, 5, 6; Rodrigo *et al.*, 2004; Alquezar *et al.*, 2008). The chromoplastic expression of *Csβ-LCY2* is also reinforced by the *in silico* expression analysis through 182 cDNA libraries generated from diverse citrus tissues, developmental stages and species. All the ESTs corresponding to *Csβ-LCY2* were identified in libraries from fruit or flower tissues and only one EST was identified in a library from green senescent ovaries (see Supplementary Table S1 at *JXB* online). This last finding could be explained by the high rate of ethylene production generating senescent citrus fruits (Gomez-Cadenas *et al.*, 2000; Katz *et al.*, 2004) since, as happens with most carotenogenic genes (Rodrigo and Zacarias, 2007), ethylene stimulates the expression of *Csβ-LCY2* (Fig. 3). These results strongly suggest that *Csβ-LCY2* plays a key role in the carotenogenesis of citrus fruits, by redirecting the flux of carotenes into the β,β-branch to lead to the accumulation of xanthophylls characteristic of orange fruit ripening.

The presence of two *β-LCY*s genes, one with a chromoplast-specific expression during fruit maturation, has previously been reported in other carotenogenic fruits. The *CYC-B* gene from tomato is transiently expressed in fruit and also detected in flower petals whereas it is undetectable in roots, leaves, and stem (Ronen *et al.*, 2000). The overexpression of this gene in tomato promotes the formation of β-carotene in the fruit while in antisense plants or defective mutants results in higher levels of lycopene in chromoplastic tissues without affecting carotenoid composition in vegetative tissues (Ronen *et al.*, 1999, 2000). In pepper, besides β-LCY, the capsanthin-capsorubin synthase (CCS), which also has β-cyclase activity, participates and has a key role in the cyclization of lycopene during fruit coloration (Hugueney *et al.*, 1995). Moreover, it has also been suggested that the orthologous *CYC-B* gene from watermelon might be an essential colour determinant

Table 3. Changes in the nucleotides and amino acid sequences between the alleles *β-LCY2a* and *β-LCY2b*

Change position		*β-LCY2 a/b*	
		Nucleotide (AA)[a]	
Nucleotide	Amino acid	a	b
79	26	C (P)	A (H)
89	29	C (S)	T (S)
202	67	C (V)	G (E)
217	72	C (D)	A (V)
287	95	T (P)	A (P)
327	109	A (S)	G (G)
330	110	A (V)	T (I)
420	140	T (V)	C (I)
553	184	A (S)	T (L)
560	186	G (G)	A (G)
565	188	A (K)	G (R)
590	196	A (H)	G (H)
635	211	G (G)	A (H)
695	231	G (E)	T (D)
776	258	G (D)	A (D)
953	317	A (S)	G (R)
1055	351	C (P)	T (P)
1070	356	C (A)	G (A)
1077	359	A (G)	C (S)
1092	364	A (I)	G (V)
1101	367	A (A)	G (S)
1124	374	T (R)	C (R)
1145	381	C (A)	T (A)
1166	388	G (E)	T (E)
1347	449	G (Y)	A (H)
1445	481	G (L)	C (F)
1467	489	G (V)	C (L)

[a] AA, amino acid.

(Tadmor *et al.*, 2005). Therefore, the involvement of a second chromoplastic-specific *β-LCY* in the regulation of carotenoid composition appears to be a frequent mechanism in carotenogenic fruits. Interestingly, phylogenetic analysis of the citrus *β-LCY2* showed that it is more closely related to chromoplastic cyclases (*CYC-B* and *CCS*) than to the previously isolated *β-LCY1* from citrus (Fig. 2B). Evolutionarily, a common origin by duplication of an ancestral *β-LCY* has been proposed for chromoplast-specific β-cyclases (Bouvier *et al.*, 2000; Krubasik and Sandmann, 2000) in agreement with the intronless structure of the genomic sequences of lycopene β-cyclases from tomato, pepper and citrus (Deruere *et al.*, 1994; Ronen *et al.*, 2000).

Despite the moderate similarity between *Csβ-LCY2* and *Csβ-LCY1* (53% identical), several structural and functional domains previously defined in other plant β-LCYs were found in the β-LCY2 sequence (Hugueney *et al.*, 1995; Cunningham *et al.*, 1996; Fig. 2A). For example, domains proposed as essential for lycopene cyclase activity (Bouvier *et al.*, 1997) (Fig. 2A) were highly conserved. In agreement with previous studies that have demonstrated the peripheral association of the β-LCY1 from *C. sinensis* to the surface of chloroplasts (Inoue *et al.*, 2006), *Csβ-LCY2* also contains a predicted transit peptide for plastid import suggesting a similar location.

Interestingly, two different alleles of *β-LCY2*: *β-LCY2a* and *β-LCY2b* were identified in the three *Citrus* species analysed, *C. sinensis*, *C. paradisi*, and *C. clementina* (Table 3; see Supplementary Table S1 at *JXB* online). This finding was not surprising, as the existence of more than one allele for other carotenogenic genes in *Citrus*, such as *PSY*, *PDS*, *β-LCY*, and *ε-LCY* has been reported (Fanciullino *et al.*, 2007). The presence of diverse alleles of *CYC-B*, the orthologous *β-LCY2* gene of tomato, has also been described (Ronen *et al.*, 2000). The activity of both citrus *β-LCY2* alleles was assayed using a lycopene-accumulating strain of *E. coli* and although *β-LCY2a* and *β-LCY2b* encode 96% identical proteins, β-LCY2a catalyses the β-cyclization of lycopene while β-LCY2b is mostly devoid of this activity (Fig. 4). The high degree of similarity in the characteristic motifs over plant β- and ε-LCYs, and in other related enzymes such as CCS, suggests that slight alterations in the sequence of the *β-LCY2b* allele could strongly affect its activity. A good example for that has been reported in lettuce (Cunningham and Gantt, 2001), in which a change in one single amino acid in ε-LCY determines the ability of introducing one or two ε-rings in the molecule of lycopene, or even to abolish the cyclase activity. In the present investigation, 16 amino acid changes were detected between *β-LCY2a* and *β-LCY2b* sequences that presumably could alter the β-cyclase activity (Table 3). Among these amino acid substitutions, three of them are located in evolutionarily conserved domains. Changes in the amino acids 67 and 72 involve a change in the polarity of the amino acid (Val to Glu and Asp to Val, respectively) in a motif conserved in all plant β-LCY which is located in the predicted transit peptide. The replacement of Gly by Ser at the position 359 in *β-LCY2b* might result in a more dramatic alteration of β-cyclase activity since Gly is conserved in all β-LCY so far characterized, including those from photosynthetic algae and bacteria. These amino acid alterations are likely to be involved in the loss of activity found for the protein coded by the *β-LCY2b Citrus* allele. However, the impact of the different amino acid substitutions on lycopene β-cyclase activity requires further characterization.

Lycopene accumulation is an atypical feature in most citrus fruits, since it has only been reported in few species. However, it is an intermediary metabolite in the biosynthesis of β,β-xanthophylls, the most abundant carotenoids in ripe orange and mandarin fruit (Table 2; Kato *et al.*, 2004; Rodrigo *et al.*, 2004; Fanciullino *et al.*, 2006; Xu *et al.*, 2006; Matsumoto *et al.*, 2007). Recently, two orange mutants, Hong Anliu and Cara Cara, which accumulate lycopene in the pulp, have been characterized and an up-regulation of early carotenogenic genes and those from the MEP pathway, respectively, have been proposed to be responsible for their particular carotenoid composition (Liu *et al.*, 2007; Alquezar *et al.*, 2008). In both orange mutants, accumulation of lycopene occurs without affecting the

normal content and composition of xanthophylls with respect to the parental fruit. Based on the carotenoid profile, it has been proposed that the mechanism responsible for lycopene accumulation in grapefruits is different from that in red orange mutants (Xu *et al.*, 2006). Despite the commercial relevance and the extensive consumption of red grapefruit, the molecular basis of lycopene accumulation has not yet been investigated. In order to elucidate this process, the expression profiles of eight genes of the carotenoid biosynthetic pathway, including the chromoplast-specific *β-LCY2*, were analysed in the flavedo and pulp of Star Ruby red grapefruit and Navel orange during ripening (Figs 5, 6). In the pulp of mature Star Ruby, the massive accumulation of lycopene is accompanied by an increase in linear carotenes and β-carotene, and almost a completely absence of xanthophylls (Table 2). A simple hypothesis to explain this phenotype considers a blockage of the carotenoid pathway at the level of β-LCY and β-CHX (Xu *et al.*, 2006; Fanciullino *et al.*, 2007). Interestingly, in Star Ruby a substantial reduction in the expression of *β-LCY2* and *β-CHX*, but not in *β-LCY1* was observed, and was more considerable in the pulp than in flavedo (Figs 5, 6). In addition, it was also observed that the non-functional *β-LCY2b* allele was preferentially expressed in Star Ruby grapefruit, which would decrease the effective lycopene β-cyclase capability of this genotype. Together, these alterations may provide the molecular basis to explain the accumulation of lycopene in red grapefruits; a reduced expression of the *β-LCY2* and *β-CHX* genes, and the preferential transcription of the non-functional *β-LCY2b* allele. It is interesting to note that the pulp of Star Ruby also accumulates β-carotene. This effect may be explained by the reduced expression of *β-CHX* and the β-cyclization of lycopene provided by β-LCY1, whose expression and functionality in Star Ruby fruit tissues are similar to those of Navel oranges (Figs 5, 6). Also, these results indicate that β-LCY1 is not able to compensate for the reduced β-LCY2 activity, thus suggesting that β-LCY1 is not efficiently contributing to redirect the flux towards the β,β-branch in chromoplastic tissues.

Star Ruby is one of the more intense red-coloured grapefruit, especially in the pulp, and was therefore selected for this study. There are, however, wide collections of red-pigmented grapefruit cultivars exhibiting colour singularities ranging from pale pink to deep red (Rouseff *et al.*, 1992). Early investigations reported that these colour variations are due to different accumulations of lycopene and β-carotene (Khan and Mackinney, 1953; Ting and Deszyck, 1958; Gross, 1987; Rouseff *et al.*, 1992; Xu *et al.*, 2006). In other plant species, is has been reported that polymorphism in a *LCY* gene may explain the natural variation in colour in different tissues. Hence, polymorphisms in *ε-LCY* are related to β-carotene and β-cryptoxanthin content in kernels in different maize cultivars (Harjes *et al.*, 2008) and polymorphisms in *β-LCY* co-segregated with flesh colour in watermelon cultivars (Bang *et al.*, 2007). Since the *β-LCY2* gene appears to be a critical regulatory step in the biosynthesis of carotenoids in *Citrus* fruits, it is temping to speculate that the genetic colour variability found in red-pigmented grapefruits might be due either to differences in *β-LCY2* transcript accumulation or/and to relative expression between *β-LCY2a*/*LCY2b* alleles. Even though further work is needed, these results suggest that additional regulatory mechanisms of carotenoid accumulation, based on the presence of two functionally different alleles with different transcript accumulation, are important in determining carotenoid composition in *Citrus* fruits, as it has been postulated previously. It has been suggested that the β-cyclization of lycopene is the step involved in the differentiation of red grapefruits and pummelos (Fanciullino *et al.*, 2006, 2007). *β-LCY1* is a single-copy gene and grapefruit is the only specie containing the two pummelo alleles of this gene, but orange-coloured *Citrus* species carry one allele from pummelo and one from mandarin (Fanciullino *et al.*, 2007).

In conclusion, in this work the isolation and functional characterization of a novel chromoplast-specific *Csβ-LCY2* from *Citrus*, highly induced in the flavedo and pulp of Navel oranges during fruit maturation is reported. *Csβ-LCY2* appears to play a key role in the regulation of carotenoids biosynthesis, redirecting the flux of carotenes into the β,β-branch of the pathway to lead to the accumulation of xanthophylls. Two alleles of the gene have been isolated, one with a higher conversion of lycopene to β-carotene in *E. coli* and the other almost devoid of activity. In the red grapefruit Star Ruby, accumulation of lycopene during maturation was associated with a substantial reduction in the expression of *β-LCY2* and *β-CHX* genes with respect to Navel oranges. Moreover, Star Ruby grapefruit predominantly expressed the non-functional *β-LCY2* allele during fruit ripening whereas fruit tissues of Navel oranges preferably expressed the functional allele. It is suggested that the presence of diverse alleles of the *β-LCY2* gene, encoding enzymes with altered activity, with different transcript accumulation may be an additional regulatory mechanism of carotenoid synthesis involved in the accumulation of lycopene in red grapefruits.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Identification of *β-LCY2a* and *β-LCY2b* alleles in Citrus cDNA libraries.

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