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Biochemical and Molecular Analysis of Carotenoid Biosynthesis in Flavedo of Orange (*Citrus sinensis* L.) during Fruit Development and Maturation

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Changes in carotenoid content and composition and expression of carotenoid biosynthetic genes were analyzed in the flavedo of sweet orange (*Citrus sinensis* L. Osbeck, cv. Navelate) fruit during development and maturation. Lutein and all-*E*-violaxanthin were the major carotenoids in chloroplast-containing tissues. During fruit coloration, phytoene, β-cryptoxanthin, zeaxanthin, and mainly (9*Z*)-violaxanthin progressively accumulated, and a large proportion of apocarotenoids was also found in the flavedo of full-colored fruits. We have cloned partial and full-length cDNAs corresponding to genes involved in early condensation and desaturase reactions [phytoene synthase (PSY), phytoene desaturase (PDS), and ζ-carotene desaturase (ZDS)], coupled redox reaction (plastid terminal oxidase), cyclizations [β-lycopene cyclase (β-LCY) and ϵ-lycopene cyclase (ϵ-LCY)], hydroxylation [β-carotene hydroxylase (β-CHX)], and epoxidation [zeaxanthin epoxidase (ZEP)] and analyzed their mRNA accumulation in the flavedo of fruits during development and ripening as compared with those of leaves. Collectively, the results indicated that PDS gene expression correlated with carotenoid content in developing fruit and that up-regulation of PSY and ZDS genes at the onset of fruit coloration would enhance the production of linear carotenes and the flux into the pathway. The shift from the β,ϵ-branch to the β,β-branch of the pathway that originates the changes in carotenoid composition during fruit coloration may be explained by a down-regulation of ϵ-LCY and by the increase of the β-CHX transcript.

KEYWORDS: Carotenoids; *Citrus sinensis* fruits; gene expression; maturation

INTRODUCTION

The external color of *Citrus* fruits is one of the main attributes of quality and a major parameter for consumer acceptance. The development of the *Citrus* peel color is the result of coordinated changes in carotenoid content and composition and chlorophyll degradation (*1*). In the past, content and carotenoid composition in fruits of different *Citrus* cultivars were extensively studied, showing that the peel of mature fruits is one of the richest and more complex sources of carotenoids in plants (*2*, *3*).

The first committed step of carotenoid biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (C_{20}) catalyzed by PSY. Phytoene (C_{40}) undergoes four desaturation reactions catalyzed by PDS and ZDS to form lycopene (*4*). In higher plants, the cyclization of lycopene to yield β-carotene and α-carotene is the first branching point in the pathway (*5*, *6*). The formation of β-carotene is catalyzed by a single enzyme, β-LCY, whereas in the case of α-carotene two different activities, β-LCY and ϵ-LCY, are involved. α- and β-carotene suffer sequential hydroxylations of the rings by ϵ- and β-CHXs yielding oxygenated xanthophylls such as lutein (derived from α-carotene) and β-cryptoxanthin and zeaxanthin (derived from β-carotene) (*4*, *7*). The hydroxylated β-ionone rings of zeaxanthin can be modified by ZEP to form violaxanthin via antheraxanthin (*8*). One epoxy ring of violaxanthin can be rearranged to form an allenic bond by neoxanthin synthase to generate neoxanthin (*9*). In addition to the structural enzymes, other activities are required for the pathway. Particularly, desaturation reactions are linked to a respiratory redox chain, which requires the participation of a PTOX (*10*, *11*).

The regulation of carotenogenesis in fruits has been mainly studied in tomato (*Lycopersicum esculentum*) and pepper (*Capsicum annuum*) (*6*, *8*, *12*−*14*). During tomato ripening, transcript levels of PSY-1, a fruit specific ripening isoform (*15*), and PDS increase substantially at the B stage (*16*−*18*), and concomitantly, transcripts of both β- and ϵ-LCY disappear (*19*, *20*). As a result, mature tomato fruit accumulates massive amounts of lycopene. Ripening bell pepper accumulates large quantities of two specific β-carotene-derived keto-carotenoids, capsanthin and capsorubin, which are responsible for the red color (*21*). The expression of PSY, β-CHX, ZEP, and the capsanthin−capsorubin synthase (CCS) genes is strongly induced in pepper during the transition stage from chloroplast to chromoplast, but the PDS gene induction is less clear than in tomato fruit (*12*, *14*, *22*, *23*). Therefore, differencial transcriptional regulation of carotenoid biosynthesis genes seems to be

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important in determining the amount and type of specific carotenoids that accumulate fruits during ripening.

The peel of immature *Citrus* fruits shows a carotenoid profile characteristic of chloroplastic-containing tissue, lutein being the main carotenoid (*1*, *24*, *25*). A noticeable decrease in carotenes and lutein occurs at the onset of fruit coloration, with a parallel accumulation of specific β,β-xanthophylls (*1*). The predominant carotenoid in the peel of mature sweet orange fruit is the (9*Z*)-violaxanthin (*1*, *3*). The characteristic color of some colored varieties of *Citrus* fruits (sweet orange and mandarins) is provided not solely by β,β-xanthophylls (violaxanthin and β-cryptoxanthin) but also by citrus specific C_{30}-apocarotenoids. β-Citraurin, β-citraurinene, and β-apo-8′-carotenal are the most abundant C_{30}-apocarotenoids, and their biosynthetic origin remains unsolved (*1*, *3*). Recently, the relationship between carotenoid accumulation and the expression of carotenoid biosynthetic genes during fruit maturation of three *Citrus* varieties, Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*Citrus sinensis* Osbeck), and Lisbon lemon (*Citrus limon* Burm.f.), has been reported, concluding that carotenoid accumulation in *Citrus* is highly regulated by the coordinated expression of the different carotenoid biosynthetic genes (*25*).

Among the different *Citrus* species, sweet oranges from the Navel group (*C. sinensis* L. Osbeck) are probably the most cultivated worldwide for fresh fruit consumption. The analysis of carotenoid composition in peel and pulp of Washington Navel orange was investigated in detail in the past (*26*, *27*). One of the most appreciated cultivars among the Navel group is the Navelate orange, which was originated as a spontaneous mutation on a Washington Navel tree (Vinaros, Castellon, Spain) (*28*). Navelate has advantages for Spanish citriculture, including its high fruit quality, excellent organoleptic properties, and late maturation. We selected Navelate fruits to study the accumulation and regulation of carotenoid biosynthesis in the peel during fruit development and maturation. To this goal, we isolated molecular probes from genes involved in the early biosynthetic steps of the carotenoid pathway (PSY, PDS, ZDS, and PTOX), in the cyclization branching point (β-LCY and ϵ-LCY), and in violaxanthin formation (β-CHX and ZEP), since this is the main carotenoid in the peel of ripe orange. The objective of this work has been to analyze the pattern of expression of the eight genes isolated and to understand how these changes are related to the evolution of carotenoids content and composition in the flavedo of Navel oranges during fruit development and maturation.

MATERIALS AND METHODS

Plant Material. Fruits and leaves of Navelate (*C. sinensis* L. Osbeck) at different developmental stages were harvested from trees grafted on Citrange carrizo rootstock. The experiments were conducted with adult trees grown in The Citrus Germplasm Bank at Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain).

The fruit size and color were measured in 30 fruits for each developmental stage. After determination of the fruit diameter, the color was measured using a Minolta CR-330 colorimeter on three locations around the equatorial plane of the fruit. Hunter parameters *a*, *b*, and *L* were used, and color was expressed as the *a/b* Hunter color ratio, a classical relation for color measurement in citrus fruits (*29*). The *a/b* ratio is negative for green fruits, and the zero value corresponds to yellow fruits and is positive for orange fruits. The flavedo tissue (outer colored part of the fruit peel) was obtained from the 30 fruits used for size and color measurements. Thirty young (less than 4 months old) and mature (more than 8 months old) leaves were selected. The flavedo and leaves were frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis. The data of fruit diameter and color are the means ± SD of 30 replicate samples.

Chlorophyll and Total Carotenoid Extraction and Quantification. Fruit and leaf pigments were extracted as described previously (*24*). The chlorophyll ($a + b$) content was determined by measuring the absorbance at 644 and 662 nm and calculated according to the Smith and Benitez equations (*30*). After chlorophylls measurements, the pigment ethereal solution was dried and saponified using a 10% methanolic KOH solution. The carotenoids were subsequently re-extracted with diethyl ether until the hypophase was colorless. An aliquot of the ethereal extract was used for quantification of total carotenoid content. The total carotenoid content was calculated by measuring the absorbance of the saponified extracts at 450 nm, using an extinction coefficient of β-carotene, $E^{1\%} = 2500$ (*31*). The samples were dried under N_2 and kept at −20 °C until high-performance liquid chromatography (HPLC) analysis. All operations were carried out on ice under dim light to prevent photodegradation, isomerizations, and structural changes of the carotenoids.

HPLC of Carotenoids. For HPLC analysis of the carotenoids, the peels of fruits at four developmental/maturation stages were selected as follows: (i) IG fruit harvested in July−August with an *a/b* ratio of -0.79 ± 0.01, (ii) MG fruit harvested in October with an *a/b* ratio of -0.64 ± 0.02, (iii) B fruit harvested in November with an *a/b* ratio of 0.01 ± 0.02, and (iv) FC fruit harvested in December with an *a/b* ratio of 0.64 ± 0.02. Carotenoid HPLC analysis from young and mature leaves harvested in July and December, respectively, was also carried out.

The samples were prepared for HPLC by dissolving the dried residues in MeOH:acetone (2:1, v/v). Chromatography was carried out with a Waters liquid chromatography system equipped with a 600E pump and a model 996 photodiode array detector and Millenium Chromatography Manager (version 2.0) (Waters, Barcelona, Spain). A C_{30} carotenoid column (250 mm × 4.6 mm, 5 μm) coupled to a C_{30} guard column (20 mm × 4.0 mm, 5 μm) (YMC Europe GMBH, Schermbeck, Germany) were used with MeOH, water, and methyl *tert*-butyl ether. Carotenoid pigments were analyzed by HPLC using a ternary gradient elution reported in a previous work (*32*). 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. The area of each peak was obtained, and the percentage of each individual carotenoid was calculated over the total area of carotenoid peaks, as integrated by the Maxplot chromatogram. Each sample was extracted twice, and two replicate injections from each extraction were performed.

The β-carotene, α-carotene, and lycopene standards were obtained from Sigma-Aldrich (Madrid, Spain). The standards β-cryptoxanthin, lutein, and zeaxanthin were obtained from Extrasynthese (Lyon, France).

Total RNA Isolation. The plant material used for total RNA isolation was the same as that used for chlorophyll and carotenoid analysis. The total RNA was extracted from the flavedo and leaves using the following method. The ground tissue (2 g) was transferred to a tube containing 5 volumes (mL/g FW) of extraction buffer [200 mM Tris-HCl, pH 8.0, 400 mM NaCl, 50 mM Na_2EDTA, 2% (w/v) Sarkosyl, 1% (w/v) poly(vinylpyrrolidone) (M_r of 40000), and 1% (v/v) β-mercaptoethanol], and 2.5 volumes of phenol was added to the homogenate, vortexed, and incubated for 15 min at 65 °C. Then, 2.5 volumes of chloroform was added, and cellular debris was removed by centrifugation (3900*g* for 20 min). After centrifugation, the aqueous phase was reextracted once with phenol/chloroform. The nucleic acids were precipitated by adding 1.5 volumes of cold ethanol and then centrifugated immediately (13000*g* for 15 min at 4 °C), and the pellet was washed with 70% ethanol. The sediment was resuspended in 7.5 mL of TESa [10 mM Tris-HCl, pH 8.0, 5 mM Na_2EDTA, and 0.1% (w/v) Sarkosyl] and was heated at 65 °C for 15 min, and then, 7.5 mL of MilliQ water and 5 mL of 12 M LiCl were added. After an overnight incubation at 4 °C, the RNA was pelleted, washed with 1.5 mL of cold ethanol 70% (v/v), centrifuged (13000*g* for 5 min at 4 °C), and resuspended with 1.25 mL of 3 M Na-acetate (pH 6.0). The RNA was pelleted by centrifugation (13000*g* for 5 min at 4 °C) and washed with 70% (v/v) cold ethanol. The pellet was resuspended in MilliQ water, and the RNA was quantified by measuring the absorbance at 260 nm.

Table 1. Primers Used in the Amplification and Length of the cDNA Fragments from Carotenoid Biosynthetic Genes of *C. sinensis* cv. Navelate Fruits[a]

gene amplified	accession no.	primer sequence	orientation	DNA length (bp)	% identity with the best score from other noncitrus proteins (accession no.)
PSY	AY204550	GGACAGATGAGCTCGTTGATGG	S	330	93% *Cucumis melo* (P49293)
		GCTGCATTGTAGACGCTCTCTG	AS		
PDS[b]	AJ319761	GTGGATCCGGTTTGAYMGRAAACTGAA	S	418	84% *L. esculentum* (P28554)[c]
		CTAAGCTTGCRCCTTCCATNGAAGC	AS		
PTOX	AY533825	GCTCTAGARASCTTTGGYTGGTGGAG	S	207	92% *C. annuum* (AF177981)
		AGAATGGCATAYCAYTTYTCKGAATTCCG	AS		
ZDS[b]	AJ319762	CAGAAGCTTCTCTGTTGCG	S	766	82% *Tagetes erecta* (AF251013)[c]
		ATTGGATCCATACTNTCNATGTAATC	AS		
β-LCY[b]	AY533826	GCTCTAGACAAGTTTCAGAGGCG	S	517	79% *Adonis palaestina* (AF321534)[c]
		CCGAATTCTTGTTCAGATGCG	AS		
ϵ-LCY[b]	AY533827	GCGAATTCGCAGCATCAGGGAAGCTA	S	421	76% *Spinacia oleracea* (AF463497)[c]
		CATCTAGACAGCACCATATGCGAGG	AS		
β-CHX	AY533828	TGGAATTCTGGGCACGATGGGCTC	S	414	93% *L. esculentum* (Q9S6Y0)
		TCTCTAGACTGATCTCCTTCTCC	AS		
ZEP	AY533829	CAAGGTGGATGCATGGCCATTGAGG	S	272	80% *Prunus armeniaca* (AF071888)
		CCACCAACTCTTCCTGGATGTGG	AS		

[a] Y, C/T; S, G/C; R, A/G; K, T/G. S, sense; AS, antisense. Underlined sequences correspond to introduced restriction sites for cloning purposes. [b] Full-length clones were isolated from *C. sinensis* Osbeck cv. Navelate. [c] Alignments based on amino acid sequence of full-length clones.

Isolation of Partial cDNA Carotenoid Biosynthesis Genes, Probe Labeling, and Northern Blot Hybridization. Partial cDNA clones of the carotenoid biosynthetic genes PSY, PDS, ZDS, PTOX, β-CHX, β-LCY, ϵ-LCY, and ZEP were obtained by RT-PCR. cDNA synthesis was performed with 1 μg of total RNA from the flavedo tissue of fruits at the B stage, except for ϵ-LCY isolation, which was performed with RNA from the flavedo of IG fruits. The reaction was carried out in the presence of 500 ng of oligo-dT, with 200 units of Superscript II Reverse Transcriptase (Gibco BRL, Karlsruhe, Germany). Carotenoid biosynthetic gene DNA fragments were obtained by PCR amplification of the cDNA using specific primers. Primer pairs for each gene were designed on the sequences of the corresponding genes available in the public sequence databases. In the cases of PDS, ZDS, and PTOX, in which the *Citrus* sequence had not been reported at the initiation of this study, degenerated primers were designed on conserved regions from homologous genes from other plants. In **Table 1**, a list of primer sequences used and the lengths of cDNA fragments obtained are indicated. Restriction sites were engineered in some primer pairs (see **Table 1**) for cloning purposes. The identity of all cDNA fragments was confirmed by sequencing.

All probes were labeled with [α-^{32}P]dATP by PCR using the Strip-EZ PCR Kit (Ambion, Cambridge, United Kingdom) following the instructions of the manufacturer. An equivalent number of counts (10^6 cpm/mL) were used for hybridization.

Samples of denatured total RNA (10 μg) were electrophoresed on 1% (w/v) agarose-formaldehyde gels and blotted onto nylon membranes (Hybond-N, Amersham-Bioscience, Barcelona, Spain) according to standard molecular procedures (*33*). Equal loading was confirmed by ethidium bromide staining and by membrane staining with methylene blue. Northern blots were prehybridized and hybridized at 42 °C to the DNA probes with ULTRAhyb hybridization buffer (Ambion) and washed following the instructions of the manufacturer. The membranes were then exposed to Kodak X-Omat SX film.

RESULTS

Evolution of Fruit Diameter, Color, Chlorophyll, and Carotenoid Contents in the Peel of Navelate Orange during Development and Ripening. From late July, orange fruit encompassed a phase of rapid development (mainly due to cell enlargement) to reach its final fruit size around late October. During this period, the color of the peel remained dark green. Thereafter, the end of the fruit development phase was coincident with the initial signal of fruit degreening (**Figure 1A**). The color of the peel changed progressively from pale green to yellow, to reach the B stage (*a*/*b* Hunter ratio 0) around middle November. The distinctive orange color of the fruit was gradually developed up to late December, with minor variations thereafter (**Figure 1A**). During the period of fruit development, the chlorophyll content declined (from 275 μg/g FW in IG fruit to 120 μg/g FW in MG fruit), and at the onset of fruit coloration,

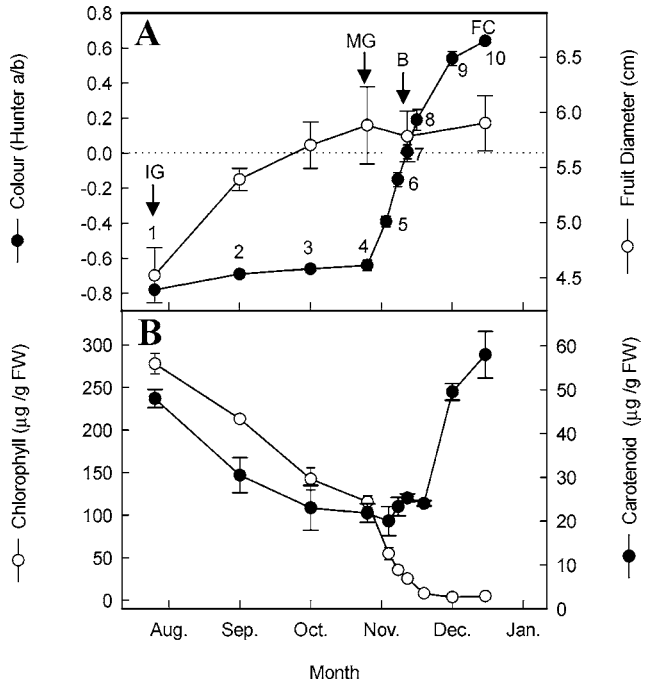

Figure 1. Evolution of fruit diameter and peel color (**A**) and chlorophyll and carotenoids content in the peel (**B**) during development and maturation of Navelate fruits (*C. sinensis* L. Osbeck). Fruit color is expressed as an *a*/*b* Hunter ratio. The dotted line indicates the color index at the color brake. The numbers indicate the samples used for analysis of gene expression (see **Figure 3**), where 1 corresponds to IG, 4 corresponds to MG, 7 corresponds to B fruits, and 10 corresponds to FC fruits. The data of fruit diameter and color are the means ± SD of 30 replicate samples. The data of chlorophyll and carotenoid contents are the means ± SD of three measuraments.

Table 2. Chromatographic and Spectroscopic Characteristics of the More Relevant Carotenoids in the Fruit Peel of *C. Sinensis* L. Osbeck Navelate Variety

	observed			literature		
t_R (min)	tentative identification[a]	λ_{max} (nm)	peak ratio[b]	λ_{max} (nm)	peak ratio[b]	ref
14.36	apocarotenoid	405, 429, 457	5	405, 430, 460	10	*43*
15.03	all-*E*-violaxanthin	415, 438, 468	92	414, 442, 472	98	*43*
15.70	neoxanthin	412, 434, 463	90	412, 434, 464	85	*43*
19.13	apocarotenoid (β-citraurin)	458	0	456	0	*26*
20.23	(9*Z*)-violaxanthin	*cis* 325, 411, 434, 463	95	*cis* 326, 416, 440, 465	98	*43*
23.30	lutein[a]	418, 444, 472	65	421, 445, 474	60	*43*
24.23	apocarotenoid	467	0			
26.14	zeaxanthin[a]	430, 450, 478	35	428, 450, 478	26	*43*
29.00	phytoene	273, 285, 300	10	276, 286, 297	10	*43*
30.44	α-cryptoxanthin	419, 445, 472	72	421, 445, 475	60	*43*
31.53	phytofluene	329, 346, 364	71	331, 348, 367	90	*43*
33.61	β-cryptoxanthin[a]	423, 450, 479	30	428, 450, 478	27	*43*
38.70	α-carotene[a]	420, 445, 472	62	422, 445, 473	55	*43*
41.90	β-carotene[a]	426, 451, 473	31	425, 450, 477	25	*43*
43.12	*Z*-β-carotene	*cis* 340, 422, 446, 473	34	*cis* 342, 424, 446, 472	33	*26*

[a] Identified using authentic standards. [b] Peak ratio is % III/II for carotenoids (*44*).

chlorophyll was rapidly degraded to minimum levels (below 10 μg/g FW) at the B stage. The content of total carotenoids followed two phases: first, a decline during the increase of fruit size, followed by a minor increase during the 2 weeks after the onset of fruit coloration. After this lag period, the total carotenoid increased to a maximum content in FC fruit (59 μg/g FW) (**Figure 1B**).

Identification of Carotenoids and Changes in Their Proportion in the Flavedo of Fruit during Development and Maturation. The composition of carotenoids was analyzed in the flavedo of Navelate oranges at four developmental stages: IG, MG, B, and FC fruit (samples 1, 4, 7, and 10 of **Figure 1A**). For comparison, the carotenoid composition was also determined in fully developed young and mature leaves. By HPLC analysis using a C_{30} column and by comparison of the spectra and the retention times with those of authentic standards, when available, or by the data reported in the literature, we were able to identify more than 20 individual carotenoids (*24*). In **Table 2**, the characteristic chromatographic and spectroscopic features of the more relevant carotenoids found in the flavedo of Navelate oranges are summarized.

The carotenoid composition of flavedo from IG fruits resembled that of leaves although the percentages of phytoene and α- and β-carotene were largely higher in leaves. The main carotenoids identified in the flavedo of IG fruits were lutein and violaxanthin, which accounted for about 50 and 20%, respectively, of total carotenoids, and minor proportions of other carotenoids characteristics of chloroplastic tissue (neoxanthin, α- and β-carotene, and trace amounts of α-cryptoxanthin, zeaxanthin, and phytoene). It is interesting to notice that all of the violaxanthin detected in the flavedo at this stage was as the all-*E*-isomer.

At the MG stage, the proportion of phytoene was doubled, a significant amount of (9*Z*)-violaxanthin accumulated, the percentage of zeaxanthin increased, and the proportion of green tissue carotenoids was maintained (all-*E*-violaxanthin and β-carotene) or decreased (α-carotene, lutein, and neoxanthin). As the flavedo became chromoplastic-containing cells (B stage), the carotenoid composition was substantially different from that of green flavedo (**Figure 2**). The proportion of all-*E*-violaxanthin was similar to that of green fruits, and some chloroplastic carotenoids decreased (lutein) or completely disappeared (α- and β-carotene and neoxanthin). Concomitantly, the content of phytoene and zeaxanthin increased, β-cryptoxanthin reached a peak, and apocarotenoids began to accumulate. The percentage of apocarotenoids was calculated as the sum of the percentage of three compounds tentatively identified as apocarotenoids (**Table 2**). At the B stage, the proportion of violaxanthin was more than 60% of the total carotenoids and the (9*Z*)-isomer accounted for more than twice that of the all-*E*-isomer. The flavedo of FC fruits contained violaxanthin as the main carotenoid in a ratio 9*Z*:all-*E* of 4:1. The percentage of phytoene reached 1.5% of total carotenoids, β-cryptoxanthin was substantially reduced, and apocarotenoids represented 11% of the total carotenoid content (**Figure 2**). Moreover, in the flavedo of FC fruits, trace amounts of phytofluene were also detected, suggesting that stimulation of the carotenogenesis occurred at very early steps of the pathway.

The total carotenoid content in mature leaves was much higher (210 ± 24 μg/g FW) than in young leaves (80 ± 8 μg/g FW). HPLC analysis of the carotenoid composition revealed that the same carotenoids were present in both tissues. Lutein was the main oxygenated xanthophyll and in a similar proportion (around 50%) in young than in mature leaves. Violaxanthin accounted for up to 20% of the total carotenoids in both young and mature leaves, and only the all-*E*-isomer was detected. While the percentage of α-carotene increased during leaf development, those of phytoene and β-carotene were lower in mature leaves. It is interesting to note that due to the relatively high amount of β-carotene accumulated in leaves a small proportion of the *Z*-isomer was found.

Expression Analysis of Carotenoid Biosynthetic Genes in the Flavedo of Fruit during Development and Maturation. An RT-PCR-based strategy was adopted to isolate cDNAs for carotenoid biosynthetic genes from the flavedo of Navelate oranges. cDNAs corresponding to genes involved in early condensation and desaturase reactions (PSY, PDS, and ZDS), coupled redox reaction (PTOX), cyclizations (β-LCY and ϵ-LCY), hydroxylation (β-CHX), and epoxidation (ZEP) were isolated (**Table 1**). For PDS, ZDS, β-LCY, and ϵ-LCY, full-length sequences were obtained by a RACE-PCR strategy. Comparison of the deduced protein sequences with other noncitrus species displayed an identity ranking from 76 to 93% and higher than 95% with those from other *Citrus* (**Table 1**).

To determine the patterns of mRNA accumulation of the eight carotenoid biosynthetic genes isolated during fruit development and ripening, the total RNA from flavedo of fruits at 10 different

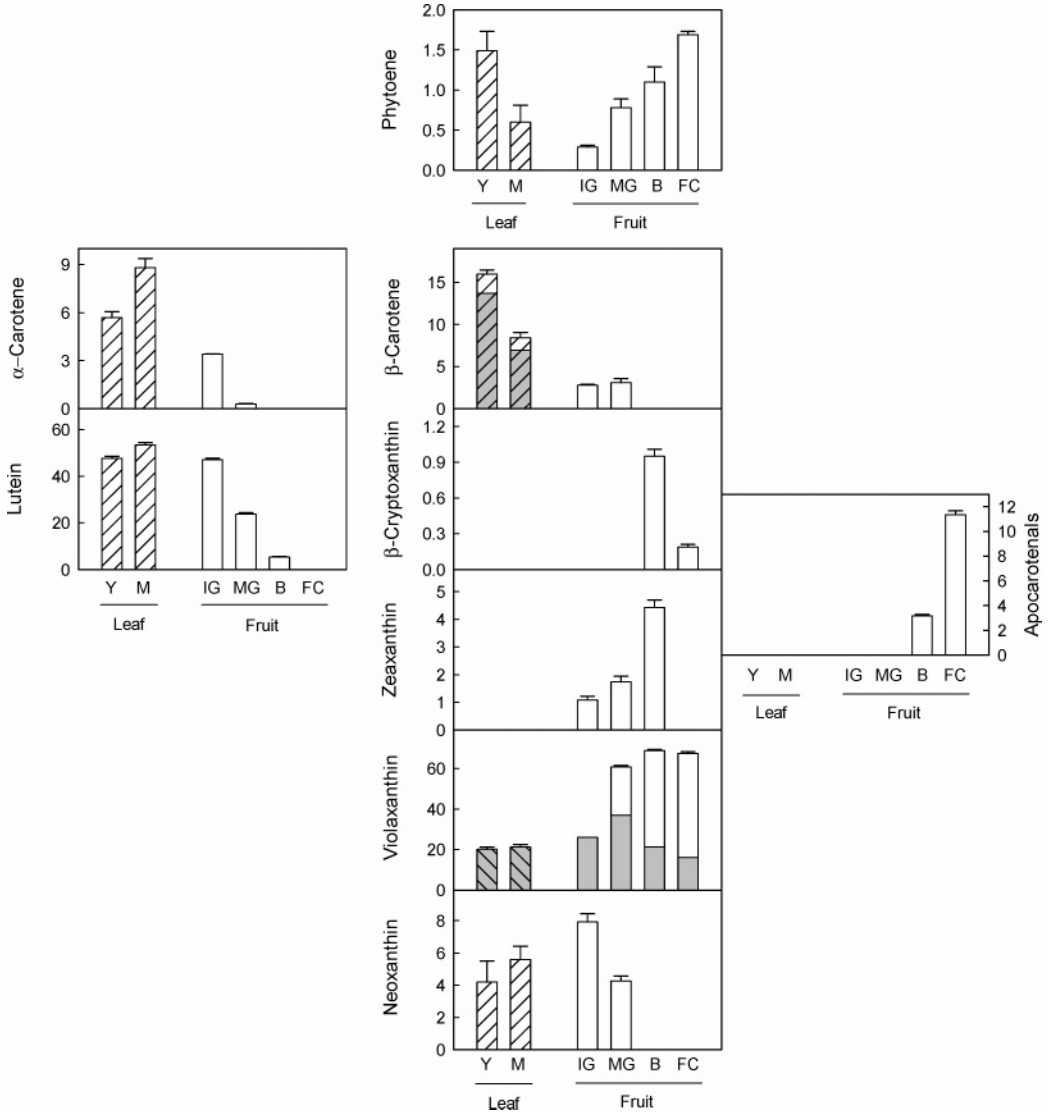

Figure 2. Distribution of carotenoids as a percentage of the total in young (Y) and mature (M) leaves (hatched bars) and in the peel of fruit at the IG, MG, B, and FC stages of Navelate oranges (*C. sinensis* L. Osbeck). The plots were arranged following the carotenoid biosynthetic sequence in the pathway. When *E*- and *Z*-isomers of a particular carotenoid are identified, the proportion of *E*-isomer is indicated in gray color. The carotenoid content is expressed as a percentage of total area of carotenoid peaks based on a Maxplot chromatogram. The values are means ± SD of at least three measurements. Note the different percentage scale for each carotenoid.

points was prepared (see **Figure 1A**). For comparison purposes, mRNA from leaves at two developmental stages was also analyzed.

Northern analysis revealed that accumulation of the mRNAs corresponding to the carotenogenic genes studied during fruit development and ripening can be grouped in at least four different patterns (**Figure 3**). First, PSY, ZDS, PTOX, and β-CHX genes followed a continuous increase of expression during fruit development and ripening. No transcript or a very low signal was detected in IG fruits, to further increased in MG fruits, just before the onset of fruit coloration, and then remained at a high level (PTOX and ZDS) or even increased further (PSY and β-CHX) toward the FC stage. A second profile was observed for the PDS gene. The transcript showed a moderated signal at the IG stage that transiently decreased until the MG stage to accumulate again as coloration of the peel progressed. A third pattern of expression was attributed to β-LCY and ZEP genes whose transcripts were consistently present through the whole process of development and ripening but at relatively low levels as compared to other carotenogenic genes. Finally, a fourth pattern of transcript accumulation was observed for the ϵ-LCY gene. The highest expression of ϵ-LCY gene was detected in the flavedo of IG fruits that rapidly declined as fruit developed. ϵ-LCY mRNA was maintained at a low level until the fruit reached the B stage, and thereafter, the transcript was hardly detectable (**Figure 3**).

It is interesting to note that mRNA accumulation of PDS, β-LCY, ϵ-LCY, and ZEP genes was much lower than that of PSY, ZDS, PTOX, and β-CHX, since membranes hybridized to their probe had to be exposed for much longer periods (5−10 times more) to obtain a detectable signal in the films.

The most noticeable difference in the expression of carotenoid biosynthetic genes between young and mature leaves was that accumulation of PDS, ϵ-LCY, and ZEP mRNAs was clearly lower in the latter. Accumulation of other transcripts was similar in both tissues, and it should be noticed that the mRNA of β-CHX was barely detected in both leaf samples.

DISCUSSION

The peel of mature orange fruit has a very complex carotenoid complement and up to 23 different carotenoids have been

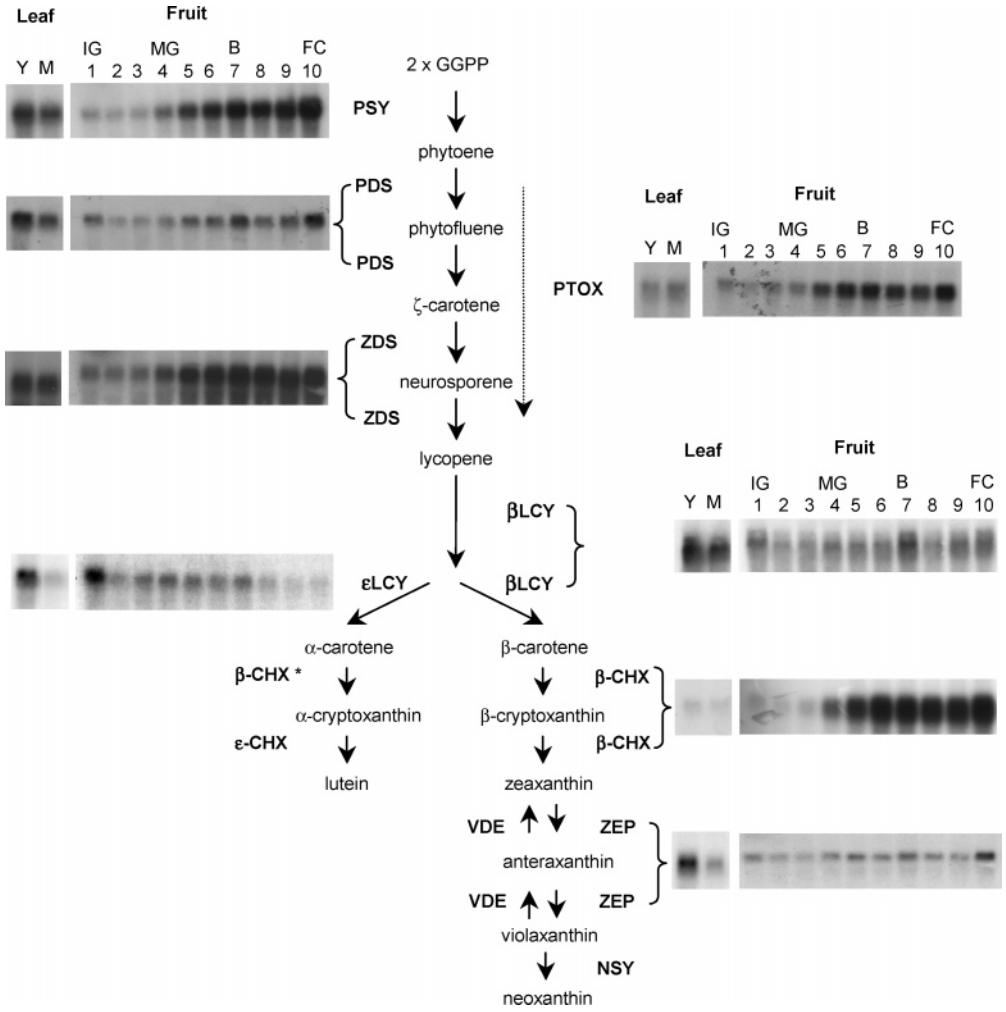

Figure 3. Accumulation of mRNAs from carotenoid biosynthetic genes in young (Y) and mature (M) leaves and in the peel of fruit during development and ripening of Navelate oranges (*C. sinensis* L. Osbeck). Fruit samples from 1 to 10 correspond to the developmental and maturation stages indicated in **Figure 1**. Northern blot analyses for each gene are arranged with the corresponding metabolic step in the pathway.

identified (*24*). However, as many of them were present in trace amounts, and analysis of only seven carotenoids (phytoene, α- and β-carotene, lutein, β-cryptoxanthin, zeaxanthin, and violaxanthin) and apocarotenoids accounted for more than 80% of the carotenoid composition at all stages analyzed, thus providing a reliable estimation of the main fluctuations in the pathway during fruit ripening (**Figure 2**).

The evolution of carotenoid content in peel during development and ripening of the Navelate orange followed a pattern that displays a minimum at the end of the cell enlargement period. During the transition from chloroplasts to chromoplasts, carotenoids accumulated at a slow rate, and once the fruit has reached the B stage, a massive accumulation of carotenoid takes place (**Figure 1**). In Satsuma mandarin and Valencia orange (*25*, *34*, *35*), and in other fruits such as tomato (*16*) and pepper (*23*), accumulation of carotenoids during fruit ripening is coincident with up-regulation of the PSY gene. However, in the peel of Navelate orange, a relatively low abundance of the PSY transcript was detected at the IG stage, which is not in concordance with the relatively high carotenoid content at that stage (**Figures 1** and **3**). By contrast, the analysis of PDS mRNA accumulation during the development and maturation process appears to be well-correlated with the changes in total carotenoid content. The PDS gene has a moderated expression at the IG stage; afterward, the level of the transcript declined at the MG stage in parallel with the decrease in carotenoid content and then returned to be up-regulated from the MG to the FC stage, when the major increase in carotenoids takes place (**Figures 1** and **3**). Moreover, the relative lower transcript level of PDS than PSY during orange ripening may explain the progressive accumulation of phytoene observed from the IG to the FC stage (**Figure 3**). Therefore, our results suggest that during development of orange fruits, and before the onset of maturation, PDS gene expression is a key mechanism regulating the production of carotenoids. As the fruit ripens, the substantial increase in PSY and ZDS gene expression may play an important role enhancing the production of linear carotenoids into the pathway.

The potential role of a PTOX (*11*) in the redox chain linked to phytoene desaturation is evidenced by its increasing expression during ripening of pepper and tomato fruit (*36*) and by the alteration of carotenoid content in the ghost mutant of tomato, which is impaired in the corresponding gene. In this report, we show the that homologous PTOX from *Citrus* was also up-regulated during fruit ripening and that the pattern of mRNA accumulation paralleled that of the mRNAs of both carotene desaturation enzymes, PDS and ZDS (**Figure 3**).

The increase in carotenoid content during the transition from chloroplast to chromoplast was associated with a change in their composition, as the reduction in the proportion of β,ε-carotenoids (α-carotene and lutein) was concomitant with the increase of β,β-xanthophylls, mainly (9*Z*)-violaxanthin, and apocarotenoids (**Figures 1** and **2**). Transcriptional down-

regulation of ϵ-LCY, coupled with up-regulation of β-CHX (see below), appear to be the critical steps in the regulation of the shift of the metabolic branch of the pathway, which originate the considerable amount of violaxanthin and apocarotenoids of colored fruits. At the IG stage, the relatively high levels of ϵ-LCY transcript would channel the flux of earlier precursors into the β,ϵ-branch resulting in the accumulation of lutein, which is part of the photosynthetic apparatus of chloroplast. Then, as fruit ripens, the dramatic decrease in ϵ-LCY transcript redirects the carotenoid flux to the massive accumulation of β,β-xanthophylls. A similar transcript profile has been found for the ϵ-LCY gene in the peel of Satsuma mandarin, Lisbon lemon, and Valencia orange (*25*).

It is particularly noteworthy that the β-LCY gene is expressed constitutively and at a relatively low level during the development and ripening of Navelate fruit. This pattern of expression is not unexpected, as the β-cyclization of lycopene is required for both ϵ,β- and β,β-branches of the carotenoid pathway (**Figure 3**). Basal β-LCY activity also explains the accumulation of β-carotene, all-*E*-violaxanthin, and neoxanthin, all derived from the β,β-branch, in IG and MG fruits (**Figure 2**). The constitutive expression of β-LCY in Navelate orange peel during fruit development and ripening differs from the up-regulated pattern reported for this gene in the peel of Satsuma mandarin and Valencia orange (*25*).

Cyclization of lycopene is a key regulatory branching point of carotenogenesis in other plants (*5*), albeit distinctly to the manner described here. Accumulation of lycopene in tomato fruits is due to down regulation of both ϵ-LCY and β-LCY genes at the B stage (*19*, *20*). In pepper, the expression of β-LCY gene is low and constitutive during fruit maturation as occurs in Navelate (**Figure 3**). However, in pepper, the CCS gene, which also has β-cyclase activity in addition to the CCS activity, is highly induced during ripening (*37*). In red pepper fruits, it has been postulated that the massive and specific channelling of carotenes into the β,β-branch is due to the simultaneous action of β-LCY and CCS (*37*). The key role of cyclases regulating the composition of carotenoids has been also extended to flower chromoplasts (*38*).

Our results also indicated that the high up-regulation of β-CHX gene in the peel of Navelate is likely contributing to the channelling of carotenoids to the β,β-branch during fruit maturation, since its expression was highly induced at the time of the major increase in violaxanthin (**Figures 2** and **3**). Our results are similar to those obtained in pepper and Valencia orange, where this gene is strongly induced during the chloroplast to chromoplast transition (*14*, *25*). In tomato, two β-CHX genes have been identified; whereas one of them was expressed in green tomato tissues, the second gene displayed a flower specific expression (*5*). The expression of our β-CHX gene was barely detected in all chloroplast-containing tissues of Navelate (fruit peel at the IG stage and leaves, **Figure 3**). This reduced expression of β-CHX gene does not explain the high proportion of lutein that we found in green tissues, as β-CHX activity is also required for lutein biosynthesis. Thus, we hypothesize that a second gene with β-CHX activity might probably exist in Navelate green tissues, similarly to that observed in tomato.

C_{30} apocarotenoids are important compounds in orange peel coloration since they provide the characteristic reddish tint. Moreover, β-citraurin has been shown to be one of the most significant pigments determining the color of orange fruit (*3*). Although the biosynthetic origin of these citrus specific C_{30} apocarotenoids remains to be elucidated, genetic evidence and their highly regulated accumulation at the onset of fruit coloration support that they may be formed by regulated enzymatic cleavage (*39*) rather than by unspecific degradation (*40*). Our results indicate that accumulation of zeaxanthin and/or β-cryptoxanthin, the putative β,β-xanthophylls precursors, preceded the major increase in apocarotenoids (**Figure 2**). The formation of apocarotenoids at the expense of zeaxanthin and β-cryptoxanthin would also explain the reduction of these xanthophylls once the flux of carotenoids has been diverted to the β,β-branch. The enzyme/s responsible for the carotenoid cleavage to generate the citrus specific C_{30} apocarotenoids has not been yet identified, although similar reactions are catalyzed by carotenoid cleavage dioxygenases in other systems (*41*).

It is important to note the substantial accumulation of (9*Z*)-violaxanthin during orange fruit maturation, while the all-*E*-isomer was an abundant carotenoid in chloroplastic tissues. The origin of the (9*Z*)-isomer has not been yet established, but it is assumed that (9*Z*)-violaxanthin is formed by in vivo isomerization of all-*E*-violaxanthin (*3*). Whether the (9*Z*)-isomer of violaxanthin is formed by an enzyme reaction or, as previously suggested, by photoisomerization from the all-*E* and then stabilized by carotenoid-binding proteins (*42*) is not currently known.

In conclusion, in the present work, we provide an overview of the relationship between accumulation of carotenoids and expression of eight biosynthetic genes in the peel of Navelate oranges during fruit development and ripening, and a comparison of their changes in leaves. The carotenoid contents in developing and mature fruit were well-correlated with the PDS gene expression profile. Once the fruit has reached the MG stage, up-regulation of PSY and ZDS genes would enhance the production of linear carotenes and the flux into the pathway. The shift from the β,ϵ-branch to the β,β-branch of the pathway that originates the changes in carotenoid composition during fruit coloration may be explained by a down-regulation of ϵ-LCY and by the increase of the β-CHX transcript.

ABBREVIATIONS USED

B, breaker fruit; β-CHX, β-carotene hydroxylase; FC, full-colored fruit; IG, immature fruit; β-LCY, β-lycopene cyclase; ϵ-LCY, ϵ-lycopene cyclase; MG, mature green fruit; PSY, phytoene synthase; PDS, phytoene desaturase; PTOX, plastid terminal oxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase.

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