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Changes in Carotenoid Content and Biosynthetic Gene Expression in Juice Sacs of Four Orange Varieties (*Citrus sinensis*) Differing in Flesh Fruit Color

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The contribution of carotenoid composition to the color range of the fruit juice sacs of four orange varieties (*Citrus sinensis*) differing in flesh color, namely, Shamouti (normal orange color), Sanguinelli ("blood cultivar" purple color), Cara Cara navel (pink-reddish), and Huang pi Chen (yellowish color), was investigated. To this end, qualitative and quantitative analyses of carotenoid contents were first performed by high-performance liquid chromatography (HPLC) using a C_{30} column and a photodiode array detector in February, at a late developmental fruit stage. Concomitantly, transcript levels of *Dxs*, the gene controlling the first step of the MEP pathway, and six genes involved in β,β-xanthophyll biosynthesis (*Psy*, *Pds*, *Zds*, *Lcy-b*, *Hy-b*, and *Zep*) were determined in August, November, and February. Transcript level measurement was carried out by real-time RT-PCR on total RNA from juice sacs. The four orange varieties displayed different carotenoid profiles. Shamouti and Sanguinelli oranges accumulated mainly β,β-xanthophylls as expected in typically colored oranges, whereas Cara Cara navel orange accumulated linear carotenes in addition to *cis*-violaxanthin. Huang pi Chen fruit flesh orange was characterized by a strong reduction of total carotenoid content. Whereas gene expression was relatively low and similar in August (before color break) in all four varieties, in November (during color break), *Dxs*, *Zds*, *Hy-b*, and *Zep* expression was higher in Cara Cara and Huang pi Chen oranges. The β,β-xanthophyll accumulation observed in February in Shamouti and Sanguinelli oranges was apparently related to the increase of transcript levels of all measured genes (i.e., *Dxs*, *Psy*, *Pds*, *Zds*, *Hy-b*, and *Zep*) except *Lcy-b*. At this time, however, transcript levels in Cara Cara were rather similar to those found in Sanguinelli, although both showed different carotenoid compositions. The Huang pi Chen phenotype correlated with lower expression of *Dxs* and *Psy* genes. These results revealed a general pattern of transcript change in juice sacs of citrus fruit, characterized by an apparent coordination of *Dxs* and *Psy* expression and a general increase in mRNA levels of carotenoid biosynthetic genes. These transcript changes correlated well with the β,β-xanthophyll accumulation, the normal carotenoid set, observed in Shamouti and Sanguinelli oranges and suggest that the preferential accumulation of linear carotenes in Cara Cara navel and the practical absence of carotenoids in Huang pi Chen oranges were not predominantly due to changes in regulation of carotenoid biosynthetic genes at the transcriptional level.

KEYWORDS: ***Citrus sinensis*; carotenoids; gene expression; allelic variability; regulation mechanisms**

INTRODUCTION

Carotenoids are pigments that play an important role in *Citrus* fruit quality. The color of the peel and pulp is mainly due to carotenoid accumulation in chromoplasts, although other pigments such anthocyanins may also contribute to pigmentation (*1*). Moreover, carotenoids have nutritional value: some of them are pro-vitamin A (mainly β-carotene, α-carotene, and β-cryp-

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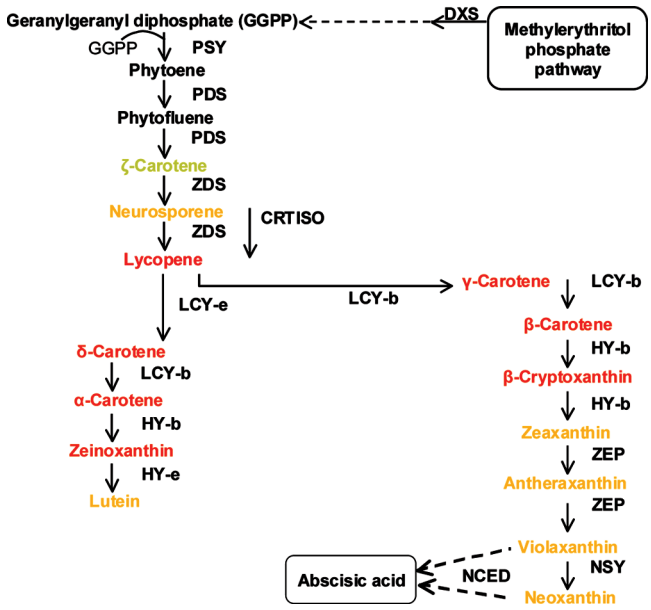

Figure 1. Carotenoid biosynthetic pathway in fruit chromoplasts. Carotenoids are written in the color they appear. Colorless carotenoids, phytoene and phytofluene, are written in black. Dashed arrows represent several steps. DXS, deoxyxylulose 5-phosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-e, lycopene ϵ-cyclase; LCY-b, lycopene β-cyclase; HY-b, β-carotene hydroxylase; HY-e, ϵ-carotene hydroxylase; ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

toxanthin in orange juices), and carotenoids have antioxidant properties (*2, 3*) that may confer to them a role in cancer and cardiovascular disease prevention (*4, 5*).

Citrus fruits generally display complex carotenoid profiles that depend on several factors including genetic factors, maturation stage, and environmental conditions. Carotenoid accumulation occurs in juice sacs of *Citrus* during fruit maturation. During the green stage of the flavedo, juice sacs of Satsuma mandarin (*C. unshiu*), orange (*C. sinensis*), and lemon (*C. limon*) were characterized by low contents in total carotenoids, the β,β-xanthophylls accumulated after the green stage (*6*). Genetic factors were also shown to play an important role in *Citrus* carotenoid composition when other sources of variation such as maturation stage, geographical origin, and cultural practices were minimized (*7*). According to our previous work, on the basis of carotenoid qualitative compositions of juices, *Citrus* species were clustered in three groups: the mandarin (*C. reticulata*) cluster, the citron (*C. medica*) cluster, and the pummelo (*C. maxima*) cluster. Orange (*C. sinensis*) varieties belonged to the mandarin cluster and at commercial maturity were characterized by β,β-xanthophyll (mainly violaxanthin and β-cryptoxanthin) accumulation in juice sacs of fruits. Orange juices contained lower β-cryptoxanthin levels than mandarins (*6–9*). However, quantitative differences were found among different orange varieties. Lee and Castle (*10*) found differences between Hamlin, Earlygold, and Budd Blood orange juices in carotenoid accumulation during the maturation period. Violaxanthin was one of the main pigments in the three varieties, but its contents changed from one variety to another. Cara Cara navel orange was characterized by the accumulation of linear carotenoids in parallel to the accumulation of violaxanthin (*11*).

The carotenoid biosynthetic pathway has been well established (*12, 13*) (**Figure 1**), but further information concerning the role of each biosynthetic step in the control of the flux of intermediates of the carotenoid pathway in *Citrus* fruits is required. Indeed, carotenoids are synthesized in chloroplasts and chromoplasts by nuclear-encoded enzymes. The immediate precursor of carotenoids (and also of gibberellins, chlorophylls, phylloquinones, and tocopherols) is the geranylgeranyl diphosphate (GGPP). The condensation of two molecules of GGPP catalyzed by phytoene synthase (PSY) leads to the first colorless carotenoid: phytoene. Precursors of carotenoids synthesized in chloroplasts and chromoplasts derive mostly from the plastidial methylerythritol phosphate (MEP) pathway (*14*). The coordination of the MEP pathway and the carotenoid pathway was shown to be involved in the regulation of carotenoid accumulation in tomato fruits (*15*). Previous study on the flavedo of *Citrus clementina* showed that the deoxyxylulose 5-phophate synthase (DXS) and PSY, the first enzymes of the MEP and carotenoid biosynthetic pathway, respectively, were involved in the control of carotenoid biosynthesis (*16*). To date, there are no data on the coordination of the MEP pathway and the carotenoid pathway during carotenoid biosynthesis in the juice sacs of *Citrus* fruit. The red carotenoid lycopene is synthesized by the action of two desaturases: phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) in four steps. Cyclization of lycopene is a branching point: one branch leads to α-carotene and the other to β-carotene. The formation of α-carotene requires the action of two enzymes, lycopene ϵ-cyclase (LCY-e) and lycopene β-cyclase (LCY-b), whereas LCY-b converts lycopene into β-carotene in two steps. According to Ronen et al. (*17*) the steps catalyzed by the two lycopene cyclases are involved in the accumulation of lycopene in tomato fruit. The synthesis of β-cryptoxanthin, one of the main pigments in the juice sacs of *Citrus* fruit, is carried out in two steps by LCY-b and β-carotene hydroxylase (HY-b). Violaxanthin is synthesized from β-cryptoxanthin in three steps: one step catalyzed by HY-b and two steps by zeaxanthin epoxidase (ZEP). The *Hy-b* and *Zep* genes should play important roles in the differential carotenoid accumulation in the juice sacs of mandarin and orange fruits (*6, 7*). Violaxanthin is converted into neoxanthin by the action of neoxanthin synthase (NSY). However, neoxanthin has not been identified in juice sacs of mandarin and orange (*18*). The catabolism of some carotenoids influenced carotenoid accumulation, mainly *cis*-violaxanthin accumulation. The enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED), which catalyzes a limiting step in abscisic acid (ABA) biosynthesis, was shown to be involved in the regulation of carotenoid accumulation in the pulp of *Citrus* fruit (*18*).

The regulation mechanisms of carotenoid accumulation during fruit maturation have been investigated by numerous studies. Works on tomato fruit suggested that the regulation of carotenoid biosynthesis was predominantly controlled at the transcriptional level (*17, 19, 20*). Lycopene accumulation would be due to the concomitant up-regulation of phytoene synthase and phytoene desaturase and down-regulation of lycopene cyclases. However, results in apricot (*Prunus armeniaca*) fruit showed that carotenoid accumulation was not correlated with the regulation of the expression of *Zds* gene involved in β-carotene synthesis (*21*). Post-transcriptional regulation, feed-back regulation by end-products, and hormonal regulation (by ethylene) were suggested as other regulation mechanisms of carotenoid accumulation. In the peel of orange fruit, during fruit maturation, the change from the β,ϵ-carotenoids to the β,β-carotenoids was explained by the down-regulation of LCY-e and the up-regulation of LCY-b and HY-b (*6, 22*). According to Ikoma et al. (*23*), the accumulation

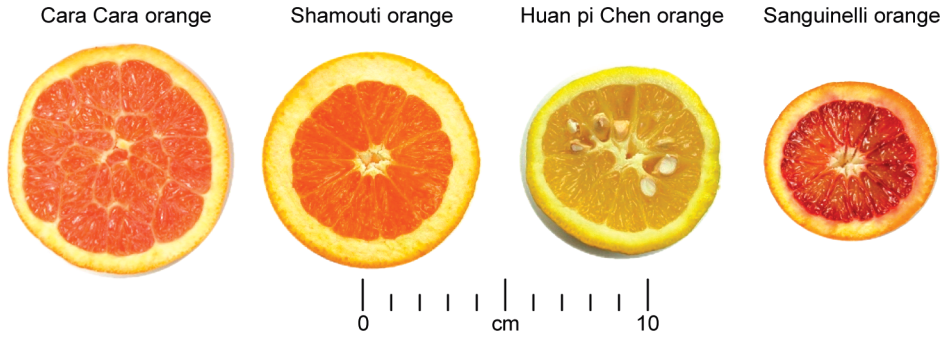

Figure 2. Fruits of four oranges differing in flesh color.

of carotenoids in both the peel and juice sacs of Satsuma mandarin (*C. unshiu*) was in agreement with the increase of *CitPsy1* transcript. The *CitPsy1* and *CitPds1* transcripts increased with the synthesis of carotenoids in the juice sacs of Satsuma mandarin, whereas in the peel, the level of *CitPds1* transcript was not coordinated with the level of *CitPsy1* transcripts (*24*). Mandarin fruits accumulated high amounts of β-cryptoxanthin in the peel and in the juice sacs during fruit maturation, but the levels of *Chx1* (or *Hy-b*) and *Chx2* transcripts were consistent in peel and juice sacs during all stages of fruit maturation, indicating that *Chx1* and *Chx2* genes were not regulated at the transcriptional level (*25*). Only one study analyzed carotenoid biosynthesis in the juice sacs of several *Citrus* varieties and species in the investigation of the expression of seven biosynthetic genes (*6*). According to these authors, in juice sacs of Satsuma mandarin and Valencia orange, a simultaneous increase in the expression of *CitPsy*, *CitPds*, *CitZds*, *CitLcyb*, *CitHyb*, and *CitZep* genes led to massive β,β-xanthophyll accumulation. Moreover, the predominant 9-*cis*-violaxanthin accumulation in juice sacs of Valencia orange would be due to a low expression of the *CitNced2* gene and therefore a low ABA synthesis (*18*). Conversely, in lemon juice sacs, the low total carotenoid content could be explained by the up-regulation of *CitNced2* and also a lower expression of the gene set involved in the production of β,β-xanthophylls (*18*). In some cases, carotenoid accumulation in fruits has been well correlated with carotenogenic gene expression, but in some *Citrus* varieties, the expression of *Pds* and *Hy-b* genes in fruit could not be related to carotenoid biosynthesis. Furthermore, only a few studies have compared several *Citrus* varieties characterized by different carotenoid compositions. Therefore, further information concerning carotenoid accumulation in *Citrus* fruit and carotenogenic gene expression is required. Although one study has analyzed carotenoid catabolism (*18*) in juice sacs of *Citrus* fruits, there are no data on MEP and carotenoid pathway interactions and their implications in carotenoid accumulation in juice sacs. Only Alós et al. (*16*) have analyzed the role of DXS in carotenoid biosynthesis in the peel of *Citrus* fruit. These authors showed that *Dxs* and *Psy* expression were not coordinated but that the highest expression of *Dxs* coincided with a peak in total carotenoid and chlorophyll abundance, suggesting that there is a competition between carotenoid and chlorophyll pathways for GGPP precursor.

The objective of this paper was to identify the regulatory mechanisms involved in the different carotenoid composition in juices of four orange varieties belonging to the same *Citrus* species but differing in flesh color. For that purpose, we harvested fruits of orange trees grown in the same area and subjected to the same cultural practices. Then, we analyzed fruit carotenoid accumulations and transcriptional regulation of the genes that lead to carotenoids biosynthesis.

MATERIALS AND METHODS

Plant Materials. Fruits of four orange varieties [*C. sinensis* (L.) Osb.] were harvested at three different developmental stages from August to February during the 2005–2006 season. The varieties used in this study were Shamouti [International Citrus Variety Numbering (ICVN) 0100299, normal orange color], Sanguinelli (ICVN 0100243, "blood cultivar" purple color and relatively high anthocyanin levels), Cara Cara navel (ICVN 0100666, pink-reddish), and Huang pi Chen (ICVN 0100567, yellowish colors) (see **Figure 2**).

Fruits were collected from adult trees grown at the germplasm collection of Station de Recherches Agronomiques of INRA-CIRAD in San Giuliano and subjected to standard cultural practices. For each genotype, at each maturation stage, 15 fruits were collected from three individual plants growing in the same field and grafted on the same rootstock variety. RNA extraction was performed from juice sacs of these four orange varieties harvested at three developmental stages: (1) on August 19 [104 days postanthesis (dpa), stage II, Bain (*26*),], with average diameters of 4.7 $\pm$ 0.2, 4.9 $\pm$ 0.4, 5.7 $\pm$ 0.2, and 5.1 $\pm$ 0.2 cm for Shamouti, Sanguinelli, Cara Cara navel, and Huang pi Chen, respectively; (2) on November 9 (186 dpa, beginning of stage III), with average diameters of 7.0 $\pm$ 0.3, 5.4 $\pm$ 0.1, 8.4 $\pm$ 0.5, and 7.2 $\pm$ 0.4 cm for Shamouti, Sanguinelli, Cara Cara navel, and Huang pi Chen, respectively; (3) on February 13 (282 dpa, stage III), with average diameters of 7.2 $\pm$ 0.6, 6.2 $\pm$ 0.1, 8.6 $\pm$ 0.4, and 8.3 $\pm$ 0.3 cm for Shamouti, Sanguinelli, Cara Cara navel, and Huang pi Chen, respectively.

Huang pi Chen produces distinctive yellow fruits. The conformity of Huang pi Chen nuclear and cytoplasmic genomes to those of *C. sinensis* was tested with simple sequence repeat (SSR) markers before carotenoid and RT-PCR analyses. Ten nuclear markers, mCrCIR07D07, mCrCIR01D06, mCrCIR06B05, mCrCIR01E02, mCrCIR01F04, mCrCIR01F08, Ci01H05, Ci02F03, Ci07C07, and C08C05 (*27*), and four chloroplastic markers, NTCP 9, CCMP6, SSCP 11, and SSCP 9 (*28*), were used. DNA extraction was performed from fresh leaves according to the method of Doyle and Doyle (*29*). PCR amplifications, polyacrylamide gel electrophoresis, and silver staining were carried out according to the method of Froelicher et al. (*27*).

Carotenoid Analysis. Carotenoid analysis was performed on the juice sacs of fruits harvested at the last developmental stage in February 2006 (282 dpa, stage III). Fruit maturity was estimated using commercial maturity indicators (*30*): juice content, soluble solid content (SSC), titratable acidity (TA), and maturity index (SSC/TA ratio) (**Table 1**). Juice content was expressed as percentage of fruit weight. TA of juices was determined by titration to pH 8.2 with 0.1 mol L^{-1} NaOH and expressed as percentage of anhydrous citric acid, and SSC was determined with a refractometer (Atago model 0−32%). Maturity index was evaluated as the SSC/TA ratio. Juice color was measured at the last developmental stage in February using a Minolta CR-310 colorimeter. An aliquot of each juice was analyzed using a plate (diameter of 5 cm) and a white background. Blank measurements were made with the plate filled with distilled water. The color parameters L^* (taking values within the range of 0–100, corresponding from black to white), a^* (negative to positive, from green to red), and b^* (negative to positive, from blue to yellow) (*31*) are presented in **Table 1**.

Carotenoids were extracted and analyzed according to the methods of Dhuique-Mayer et al. (*9*) and Fanciullino et al. (*7*). Briefly,

Table 1. Characteristics of Juices from the Four Orange Varieties in February (282 dpa)

no.	common name	diameter (cm ± SD)[b]	juice content (% ± SD)	maturity index ± SD[c]	color parameters[a]		
					L^* ± SD	a^* ± SD	b^* ± SD
1	Shamouti orange	7.2 ± 0.6	32.3 ± 1.4	7.9 ± 0.5	61.9 ± 0.2	7.7 ± 0.2	55.1 ± 0.6
2	Sanguinelli orange	6.2 ± 0.1	33.8 ± 2.5	7.1 ± 0.5	33.0 ± 1.4	32.3 ± 0.9	15.0 ± 1.7
3	Cara Cara navel orange	8.6 ± 0.4	42.4 ± 3.0	10.6 ± 0.4	58.6 ± 0.1	11.0 ± 0.4	48.4 ± 0.1
4	Huang pi Chen orange	8.3 ± 0.3	40.3 ± 2.5	6.8 ± 0.2	68.8 ± 0.2	−10.4 ± 0.2	44.6 ± 0.7

[a] Uniform color space CIELAB (*38*), L^* (taking values within the range 0–100, correspond from black to white), a^* (negative to positive, from green to red), and b^* (negative to positive, from blue to yellow). [b] SD, standard deviation. [c] Soluble solid content/titratable acidity ratio.

Table 2. Primer Pairs Used for Gene Expression Analysis by Real-Time RT-PCR

gene	primer	primer amount[a] (μL)	amplicon size (bp)
Dxs forward	5′-CGTGTTTTCAACACACCTGACG-3′	3.6	120
Dxs reverse	5′-AAGCCCCGAAGTCTTCCTCAT-3′	1.2	
Psy forward	5′-GGTCGTCCATTTGATATGCTTG-3′	0.5	111
Psy reverse	5′-CCTAAGGTCCATCCTCATTCCT-3′	0.5	
Pds forward	5′-GACAAGGAAGGGTTTCTGTCC-3′	0.5	134
Pds reverse	5′-GCTTAGAAGGACGAGGAGAAG-3′	0.5	
Zds forward	5′-CGATCCTTACATGCCCTTAC-3′	0.5	145
Zds reverse	5′-AGGTCCCTCACGGTACAAAG-3′	0.5	
Lcy-b forward	5′-CCCATGTATGACCCATCAAAG-3′	0.5	130
Lcy-b reverse	5′-TGGGAGATGGATCAATCGAG-3′	0.5	
Hy-b forward	5′-GGTGCTGGACTTGGCATTAC-3′	0.5	120
Hy-b reverse	5′-AGCGACTCTCCGGAAATAAG-3′	0.5	
Zep forward	5′-TTGGTTGATGGGATTTCTGG-3′	0.5	134
Zep reverse	5′-TCCCCAACCGCTTTAGCTAG-3′	0.5	

[a] Solution of 5 μM L^{-1}. Gene-specific primers were designed with the use of primer3 software and corresponded to *Citrus* coding sequences available in databases of *Pds* (AB046992, AJ319761, AB114657), *Zds* (AB072343, AJ319762, AB114658), *Lcy-b* (AY166796, AY533826, AB114660), *Hy-b* (AF296158, AB114661, AY533828) and *Zep* (AB075547, AB114662, AY533829). Primer pairs specific to *Dxs* and *Psy* genes were designed by Alós et al. (*16*). For all of these primers the annealing temperature was 60 °C.

carotenoids were extracted from 20 g of juice (stirred with 120 mg of $MgCO_3$) with a mixture of 35 mL of ethanol/hexane, 4:3 v/v, containing 0.1% of BHT as antioxidant. Lycopene (750 μL of solution, equivalent to 90 μg) or β-apo-8′-carotenal (150 μL, equivalent to 40 μg) was added as an internal standard. Residue was separated from the liquid phase by filtration through a filter funnel (porosity no. 2). This residue was re-extracted with 35 mL of the previous solvent and then with 30 mL of ethanol and 30 mL of hexane until it was colorless. Organic phases were transferred to a separatory funnel and successively washed with 2 × 50 mL of 10% sodium chloride and 3 × 50 mL of distilled water. The aqueous layer was removed. The hexanic phase was dried, redissolved in 20 mL of hexane, and saponified overnight at room temperature using an equal volume of 10% methanolic KOH. The sample was transferred to a separatory funnel to which 50 mL of distilled water was added to separate the layers. The hexanic layer was washed with distilled water until it was free of alkali. The methanolic KOH layer was extracted with 3 × 10 mL of dichloromethane. The extracts were pooled and washed to remove alkali. The extracts were dried using anhydrous sodium sulfate, filtered, and evaporated in a rotary evaporator. The residue was dissolved in 500 μL of dichloromethane and 500 μL of MTBE/methanol (80:20, v/v). This solution was diluted 6-fold in a MTBE/methanol mixture for three orange varieties (cv. Shamouti, Sanguinelli, Cara Car navel). Analyses were carried out under red light to avoid carotenoid degradation during extraction and saponification.

Carotenoids were analyzed by high-performance liquid chromatography using an Agilent 1100 system (Massy, France). Carotenoids were separated along a C_{30} column [250 × 4.6 mm i. d., 5 μm YMC (EUROP GmbH)]; the mobile phases were H_2O as eluent A, methanol as eluent B, and MTBE as eluent C. Flow rate was fixed at 1 mL min^{-1}, column temperature was set at 25 °C, and injection volume was 20 μL. A gradient program was performed: the initial condition was 40% A/60% B; 0–5 min, 20% A/80% B; 5–10 min, 4% A/81% B/15% C; 10–60 min, 4% A/11% B/85% C; 60–71 min, 100% B; 71–72 min, back to the initial condition for re-equilibration. Absorbance was recorded at 290, 350, 400, 450, and 470 nm using an Agilent 1100 photodiode array detector. Chromatographic data and UV–visible spectra were collected, stored, and integrated using an Agilent Chemstation plus software.

Carotenoids were identified using their retention times, absorption spectra, and co-injection with authentic standards. Standards were purchased from Extrasynthese (Genay, France): β-carotene, β-cryptoxanthin, zeaxanthin, lutein, lycopene, β-apo-8′-carotenal. Quantification of carotenoids was achieved using calibration curves with β-carotene, β-cryptoxanthin, lutein, lycopene, and β-apo-8′-carotenal with five concentrations. Correlation coefficients ranged from 0.994 to 0.998. Other carotenoids were quantified as β-carotene. Each carotenoid was quantified using area collected at 290 or 350, 400, 450, and 470 nm depending on its maximum λ. Recoveries were determined by adding an internal standard (lycopene or β-apo-8′-carotenal) before the extraction of each sample analyzed and used to correct carotenoid contents after HPLC analysis. The concentration of each carotenoid was expressed as milligrams per liter. The total contents in carotenoid pigments of juices were calculated by summing the concentrations of all compounds. The concentrations of carotenoid pigments were also expressed as relative percentage of total peak area. Analysis precision was checked from three consecutive extractions−saponifications−injections of the same sample, and coefficients of variation were ≤5%. Concentrations were expressed as the mean of the data from three extractions. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated for β-cryptoxanthin by preparing serial dilutions of this compound in the mobile phase (concentrations ranging from 1 to 10 mg L^{-1}). Calibration curves and then LOD and LOQ were determined with LOD = 3 × S/a and LOQ = 10 × S/a (where S is the standard deviation of the blank signal and a the slope of the calibration curve). An ANOVA test was performed with the GLM procedure of SAS (SAS Institute Inc., 1989) to analyze quantitative differences between orange varieties.

Total RNA Isolation and RT-PCR Analysis. Pulp of peeled fruits was immediately frozen under liquid nitrogen and kept at −80 °C until analysis. Total RNA was isolated from juice sacs as described by Manning (*32*) and cleaned up with the RNeasy Plant Mini Kit (Qiagen). DNase I treatment (RNase-Free DNase Set; Qiagen) was applied in the purification column according to the manufacturer's instructions. UV absorption spectrophotometry and gel electrophoresis were performed to test RNA quality and integrity as described by Sambrook et al. (*33*).

RT-PCR. Quantitative mRNA analyses were performed according to the method described by Alós et al. (*16*). A Roche LightCycler 2.0 instrument was used with LightCycler 4.0 software. RT-PCR was performed in a total volume of 10 μL with 50 ng of total RNA, 2.5 units of Multiscribe Reverse Transcriptase (Applied Biosystems), 1 unit of RNase Inhibitor (Applied Biosystems), 2 μL of LC FastStart DNA MasterPLUS SYBR Green I (Roche), and 2.5 μM specific primers (**Table 2**). Primer pairs for each biosynthetic gene were based on the corresponding *Citrus* coding sequences available in databases. The RT-

Table 3. Chromatographic and Spectral Characteristics of Carotenoids Found in Orange Juices

				λ_{max} (nm) observed			
no.	RT[a] (min)	tentative ID		peak I	peak II	peak III	% III/II[b]
1	17.10	*cis*-apocarotenoid[c]	cis328	408	430	458	
2	17.42	unidentified	cis328	416	439	468	76
3	18.66	unidentified		399	422	448	75
4	19.31	unidentified		400	422	448	
5	20.28	*cis*-violaxanthin[c]	cis328	412	436	464	81
6	21.41	luteoxanthin[c]		396	418	443	75
7	21.54	mutatoxanthin[c]		404	426	448	31
8	22.05	lutein[d]		422	444	472	48
9	22.29	mutatoxanthin[c]		407	428	451	
10	23.62	zeaxanthin[d]		426	450	476	17
11	24.60	*cis*-antheraxanthin[c]	cis330	417	440	468	47
12	25.75	unidentified		422	446	473	44
13	27.04	*cis*-β-cryptoxanthin[c]	cis338	420	444	470	
14	27.71	zeinoxanthin[c]		422	445	473	47
15	27.90	phytoene[c]		276	286	298	
16	30.01	β-cryptoxanthin[d]		427	450	477	20
17	30.39	phytofluene[c]		331	348	368	68
18	34.03	ζ-carotene[c]		379	400	424	90
19	34.92	α-carotene[d]		422	444	470	34
20	37.38	β-carotene[d]			452	477	12
21	49.63	*cis*-lycopene[c]	cis355	441	466	490	
22	59.44	lycopene[d]		446	472	502	71

[a] RT, retention time. [b] Spectral fine structure value calculated as the percentage of the quotient between band III and band II (λ_{max}) taking the trough between the two bands as the baseline. [c] Tentative identification by comparison with data available in the literature (*8, 22, 34–36*). [d] Identified using authentic standards.

PCR procedure consisted of 48 °C for 30 min and 95 °C for 10 min followed by 45 cycles at 95 °C for 2 s, 60 °C for 10 s, and 72 °C for 15 s. Fluorescent intensity data were acquired during the 72 °C extension step. Specificity of the amplification reactions was checked by postamplification dissociation curves and by sequencing the reaction products. To transform fluorescent intensity measurements into relative mRNA levels, a 10-fold dilution series of a RNA sample was used as the standard curve. Total RNA amounts were accurately quantified with the RiboGreen dye (Molecular Probes, Eugene, OR) and were used to normalize mRNA levels as described by Alós et al. (*16*). Values are the mean of at least three independent analyses.

Statistical Analysis. Statistical analysis was performed with the GLM procedure of SAS (SAS Institute Inc., 1989) to reveal significant differences between mRNA levels for each gene and at each developmental stage.

Correlations between the carotenoid contents (expressed as percentage of total carotenoid area in February), between the transcript levels of seven carotenoid biosynthetic genes in February, and between the carotenoid contents and the expression levels of carotenoid biosynthetic genes in February were calculated using XlStat software (Addinsoft).

Cloning and Sequencing *C. sinensis Lcy-b* Genomic DNA and Allelic Diversity Analysis. Amplification of the *Lcy-b* gene was performed by PCR using *C. sinensis* (cv. Shamouti, Sanguinelli, and Cara Cara navel) genomic DNA as template and specific primers: LCY-b2 forward, 5′-ATGGATACTGTACTCAAAACTCA-3′, and LCY-b2 reverse, 5′-TTAATCTGTATCTTGTACCAAGTT-3′. This primer pair was designed on the basis of the sequences of *Lcy-b* cDNA from *C. unshiu* and *C. sinensis* (AY166796, AY094582, AF240787, AY679167, AY679168, and AY644699). PCR was performed in a total volume of 50 μL with 1× Pfx50 PCR mix (Invitrogen), 0.2 mM dNTP, 1 μM forward and reverse primers, 5 units of Pfx50 DNA polymerase, and 100 ng of template genomic DNA. The amplification procedure was as follows: 1 cycle of 94 °C, 2 min; 35 cycles of 94 °C, 15 s; 55 °C, 30 s; 68 °C, 6 min; 1 cycle of 68 °C 5 min. The amplified fragments for each *C. sinensis* cultivar were separated by electrophoresis on 1% agarose gel, purified with GFX PCR DNA and gel band purification kit (Amersham), and cloned into the TOPO Cloning vector using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's recommendations. For the *Lcy-b* gene five clones per cultivar were sequenced. Sequencing was carried out by MWG BIOTECH (Germany) with the specific primers previously defined. Sequence alignments were carried out using the clustal W multiple-alignment procedure of Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

RESULTS

Characterization of the Four Color-Contrasted Oranges at a Late Developmental Stage in February (282 dpa). To investigate the role of the expression of carotenoid biosynthetic genes in the intraspecific variability in carotenoid composition, we analyzed four color-contrasted orange (*C. sinensis*) varieties (cv. Shamouti, Sanguinelli, Cara Cara navel, and Huang pi Chen). It is important to note that Huang pi Chen produces yellow fruits (Huang pi Chen juice is characterized by a negative CIE a^* value, positive values corresponding to reddish colors) and that the conformity of Huang pi Chen genomes to *C. sinensis* genomes was verified by SSR analyses. Analyses were performed on the juice sacs of these four orange varieties harvested at a late developmental stage in February (282 dpa). Maturity indicators (diameter, juice content, and maturity index) used to characterize the maturity stage are reported in **Table 1**. Color parameters measured on juices from these same fruits are also presented in **Table 1**. Values of color parameters were contrasted among the four oranges, reflecting differences between the varieties at this developmental stage (282 dpa). Indeed, Sanguinelli orange was characterized by the highest a^* value (32.3 ± 0.9), whereas Huang pi Chen presented the lowest value for a^* (-10.4 ± 0.2). For Cara Cara navel the juice color score of a^* value was higher compared to that of Shamouti. With regard to the juice color parameter b^* (positive values corresponding to yellowish colors), it was interesting to note that the juice of Shamouti presented the highest score (55.1 ± 0.6) and the juice of Sanguinelli the lowest (15.0 ± 1.6).

Diversity of Carotenoid Profiles within the Four Orange Varieties in February (282 dpa). Carotenoids from juices of these four oranges collected in February were analyzed by HPLC. Twenty-two carotenoid pigments were isolated and quantified. Their chromatographic and spectral characteristics are shown in **Table 3** and **Figure 3**. Lutein (peak 8), zeaxanthin (10), β-cryptoxanthin (16), α-carotene (19), β-carotene (20), and

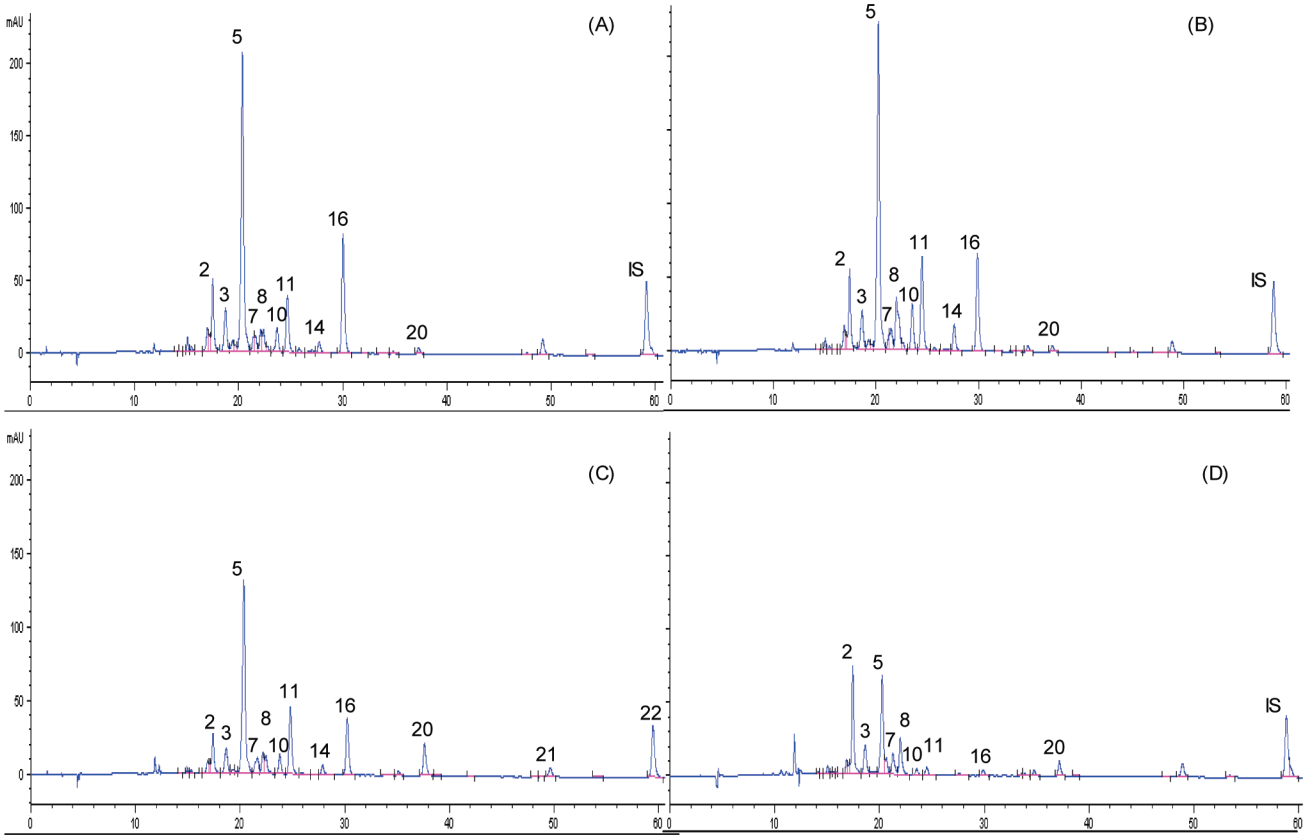

Figure 3. Chromatograms at 450 nm of carotenoids from juices of four orange varieties (**A**, Shamouti; **B**, Sanguinelli; **C**, Cara Cara navel; **D**, Huang pi Chen). Tentative peak identification is presented in **Table 3**. IS, internal standard. β-Apo-8′-carotenal and lycopene were used as internal standard in nonsaponified (chromatograms not shown) and saponified extracts, respectively. For Cara Cara navel, no internal standard was used in saponified extract. Note that the extracts for Huang pi Chen were not diluted before injection.

lycopene (22) were identified by co-injection with authentic standards. Other peaks (1, 5–7, 9, 11, 13–15, 17–18, and 21) were tentatively identified by comparison of their chromatographic and spectral characteristics with those available in the literature obtained from the same plant material (orange juices) and under the same chromatographic conditions (C_{30} column and a mixture of water, methanol, and MTBE as eluent). Four peaks (2−4 and 12) remained unidentified. Concentrations, in milligrams per liter, were calculated as the mean of at least three measurements. Total content in carotenoid pigments in juices was calculated by summing the concentrations of all individual compounds (**Figure 4**).

Carotenoid profiles from the four orange varieties were compared (**Figure 3**). No qualitative differences were found between Shamouti and Sanguinelli oranges (profiles **A** and **B** in **Figure 3**). Shamouti and Sanguinelli oranges accumulated β,β-xanthophylls: *cis*-violaxanthin (peak 5, **Figure 3**), lutein (8), zeaxanthin (10), *cis*-antheraxanthin (11), and β-cryptoxanthin (16). In addition, β,β-xanthophylls accounted for 62.9 and 59.5% of total carotenoid peak area in juices of Shamouti and Sanguinelli, respectively. *cis*-Violaxanthin and β-cryptoxanthin were the most abundant pigments in these two genotypes. Percentages of *cis*-violaxanthin were 37.4 and 34.4%, whereas percentages of β-cryptoxanthin were 15.0 and 10.2% in Shamouti and Sanguinelli, respectively. These results indicate that the difference in color between both varieties (see **Table 1**) is mostly due to the presence of anthocyanins, because the carotenoid compositions are practically identical in Shamouti and Sanguinelli. The carotenoid compositions of Cara Cara navel and Huang pi Chen oranges were different from those of Shamouti and Sanguinelli oranges (see profiles **C** and **D** in **Figure 3**). Cara Cara navel accumulated two pigments, *cis*-lycopene (21) and *all-trans*-lycopene (22), which were not detected in the other varieties. Some carotenoid pigments were undetected or detected as traces in juices of Huang pi Chen (for example, peaks 10, 11, 14, and 16 in **Figure 3**). In Cara Cara navel and Huang pi Chen oranges, proportions of β,β-xanthophylls were lower than those in Shamouti and Sanguinelli oranges. β,β-Xanthophylls represented 25.7 and 25.1% of total carotenoid peak area in juices of Cara Cara navel and Huang pi Chen oranges, respectively. Cara Cara navel presented high proportions of linear carotenes. In this orange, the main carotenoids, in relative proportions, were phytoene and *cis*-violaxanthin. The percentage of phytoene was 37.5% of total carotenoid peak area, whereas *cis*-violaxanthin represented 15.1%. *cis*-Violaxanthin, ζ-carotene, and phytoene were the main pigments in Huang pi Chen. The proportions of these carotenoids were 20.6, 9.1, and 8.5%, respectively.

Quantitative differences were also found. It is important to note that total carotenoid contents in Shamouti, Sanguinelli, and Cara cara navel oranges were much higher than in Huang pi Chen orange (see **Figure 4**). Indeed, total carotenoid contents in these genotypes were 23.40, 33.03, 47.41, and 2.51 mg L^{-1}, respectively. Consequently, *cis*-violaxanthin, which was the main carotenoid in Huang pi Chen, reached 0.52 mg L^{-1} in this genotype, whereas contents were 8.90, 11.52, and 7.35 mg L^{-1} in Shamouti, Sanguinelli, and Cara Cara navel oranges, respectively (**Figure 4**).

Transcript Levels of Seven Genes in Juice Sacs of Four Orange Varieties at Three Maturation Stages. The expression of seven genes (*Dxs*, *Psy*, *Pds*, *Zds*, *Lcy-b*, *Hy-b*, and *Zep*) involved in the MEP and carotenoid pathways was analyzed in

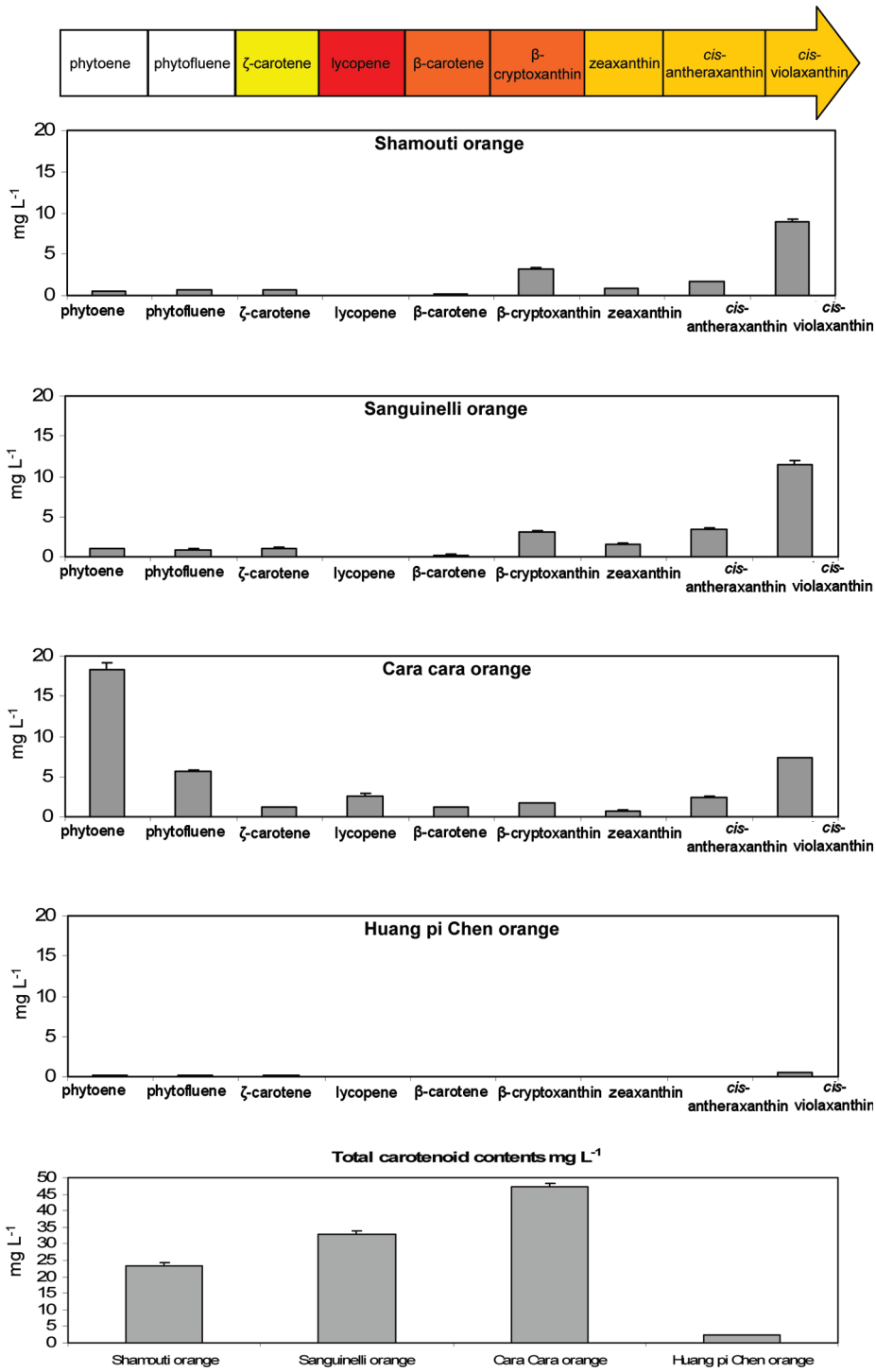

Figure 4. Carotenoid contents in the juice sacs of four orange varieties, Shamouti, Sanguinelli, Cara Cara navel, and Huang pi Chen, at development stage III. Contents are expressed in mg L^{-1}, values are means ± SD of at least three measurements. The values for total carotenoid contents were the sum of all carotenoids characterized by HPLC analysis and quantified with calibration curves and expressed in mg L^{-1}. For β-cryptoxanthin the limit of detection is 0.0046 μg and the limit of quantification 0.0152 μg.

juice sacs of four orange varieties by real-time RT-PCR. Primer pairs were defined with primer 3 software using coding sequences from *Citrus* fruits available in public databanks (**Table 2**). The expression of these genes was studied at three maturation stages [in August (104 dpa), in November (186 dpa), and in February (282 dpa)] (see **Figure 5**). Expression values were the mean of at least three measurements. Statistical analyses were performed to reveal significant differences among the four orange varieties.

Two different expression patterns were distinguished. The first one concerned the expression profiles of *Dxs* and *Psy* genes that were very similar. *Dxs* and *Psy* genes, which are involved in the biosynthesis of phytoene, the first carotenoid of the biosynthetic pathway, presented a continuous increase in expression during fruit maturation for the four genotypes. *Dxs* and *Psy* transcripts reached the highest levels at the last developmental stage in February (282 dpa): the transcripts increased 6−20- and 3−6 -fold for *Dxs* and *Psy* genes, respectively, from

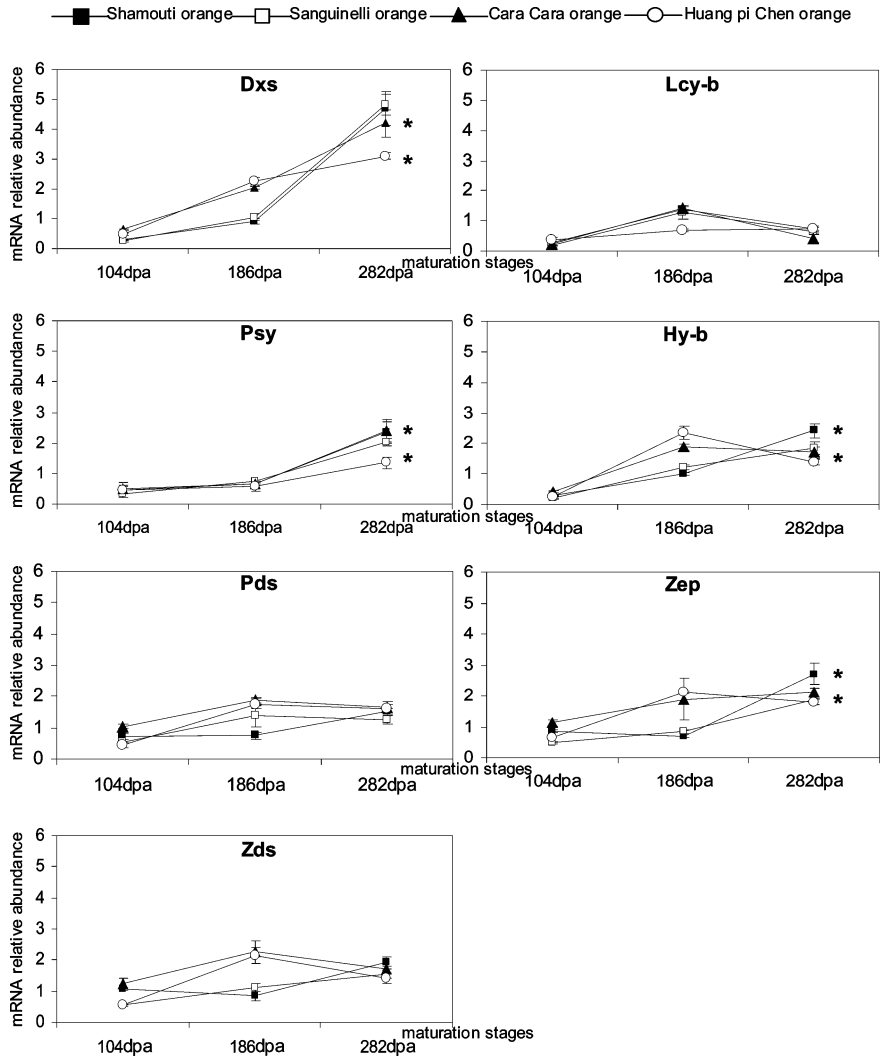

Figure 5. mRNA levels of *Dxs*, *Psy*, *Pds*, *Zds*, *Lcy-b*, *Hy-b*, and *Zep* in juice sacs of four orange varieties, Shamouti, Sanguinelli, Cara Cara navel, and Huang pi Chen, at three maturation stages, 104 days postanthesis (dpa), 186 dpa, and 282 dpa. Expression profiles were determined by real-time RT-PCR. Arbitrary units were assigned to mRNA level scales. Data are means ± SD of three measurements. Asterisks indicate statistically significant differences in mRNA levels between genotypes ($P < 0.01$ for *Dxs* and *Psy* genes and $P < 0.05$ for *Hy-b* and *Zep* genes).

104 to 282 dpa. Interestingly, a significant difference was found between the four genotypes at 282 dpa. Transcript levels at 282 dpa for both *Dxs* and *Psy* genes were much lower in juice sacs of Huang pi Chen orange when compared to the other oranges.

The second expression pattern concerned the expression profiles of *Pds*, *Zds*, *Lcy-b*, *Hy-b*, and *Zep* genes. In Cara Cara navel and Huang pi Chen oranges, transcript levels of these five genes increased in juice sacs until 186 dpa except for the *Lcy-b* gene and Huang pi Chen orange. Indeed, *Lcy-b* transcript levels did not change significantly in juice sacs of Huang pi Chen during maturation. *Pds*, *Zds*, *Hy-b*, and *Zep* transcripts increased 2−9-fold from 104 to 186 dpa in Cara Cara navel and Huang pi Chen oranges. In juice sacs of Shamouti and Sanguinelli oranges, the increase in *Pds*, *Zds*, *Hy-b*, and *Zep* expression continued from 186 to 282 dpa. Consequently, *Pds*, *Zds*, *Hy-b*, and *Zep* transcripts increased 2−10-fold from 104 to 282 dpa in Shamouti and Sanguinelli.

Relationships among the 10 carotenoid variables (expressed as percentage of total carotenoid area at 282 dpa), the mRNA levels of 7 carotenoid biosynthetic genes at 282 dpa, and the carotenoid variables and the mRNA levels were explored by means of simple correlations (**Tables 4**−**6**). Significant positive correlations ($p < 0.05$) were found between carotenoid variables: between phytoene and phytofluene and lycopene ($r = 0.969$ and 0.985, respectively) and between zeaxanthin and *cis*-antheraxanthin and *cis*-violaxanthin ($r = 0.938$ and 0.852, respectively). Significant negative correlations were also found: between β-carotene and β-cryptoxanthin, zeaxanthin, *cis*-antheraxanthin, and *cis*-violaxanthin ($r = -0.936$, -0.916, -0.941, and -0.877, respectively) (see **Table 4**). Only positive correlations were found between mRNA levels of some genes (**Table 5**). *Dxs* was correlated with *Psy* ($r = 0.809$). *Zds* was correlated with *Hy-b* and *Zep* ($r = 0.927$ and 0.971, respectively), whereas *Hy-b* was also correlated with *Zep* ($r = 0.918$). It is interesting to note that highly positive or negative correlations were found between carotenoids and mRNA levels of some genes (**Table 6**). *Dxs* was positively correlated with β-cryptoxanthin and *cis*-antheraxanthin ($r = 0.839$ and 0.947, respectively) and negatively correlated with ζ-carotene ($r = -0.907$). *Psy* was positively correlated with total carotenoid content ($r = 0.837$) and negatively correlated with ζ-carotene ($r = -0.975$). *Pds* was negatively correlated with zeaxanthin and *cis*-antheraxanthin ($r = -0.970$ and -0.873, respectively). *Hy-b* was positively correlated with β-cryptoxanthin ($r = 0.956$) and negatively correlated with β-carotene ($r = -0.821$).

Cloning and Sequence Analysis of the *Lcy-b* Gene from Cara Cara Navel, Shamouti, and Sanguinelli Oranges. To elucidate the relationship between the accumulation of lycopene

Table 4. Pearson Correlations between the 10 Carotenoid Variables (Expressed as Percentage of Total Carotenoid Area in February)

	total carotenoid	phytoene	phytofluene	ζ-carotene	lycopene	β-carotene	β-cryptoxanthin	zeaxanthin	*cis*-antheraxanthin	*cis*-violaxanthin
	1									
phytoene	0.622	**1**								
phytofluene	0.414	**0.969**[a]	**1**							
ζ-carotene	−0.860	−0.227	0.018	**1**						
lycopene	0.737	**0.985**[a]	0.914	−0.389	**1**					
β-carotene	−0.361	0.492	0.691	0.728	0.340	**1**				
β-cryptoxanthin	0.200	−0.541	−0.714	−0.665	−0.394	**−0.936**[a]	**1**			
zeaxanthin	0.206	−0.622	−0.774	−0.489	−0.506	**−0.916**[a]	0.757	**1**		
cis-antheraxanthin	0.527	−0.332	−0.541	−0.748	−0.186	**−0.941**[a]	0.765	**0.938**[a]	**1**	
cis-violaxanthin	−0.130	−0.836	−0.943	−0.342	−0.731	**−0.877**[a]	0.905	**0.852**[a]	0.718	**1**

[a] Significant correlation ($p < 0.05$).

Table 5. Pearson Correlations between the Transcript Levels of Seven Carotenoid Biosynthetic Genes in February

	Dxs	*Psy*	*Pds*	*Zds*	*Lcy-b*	*Hy-b*	*Zep*
Dxs	**1**						
Psy	**0.809**[a]	**1**					
Pds	−0.677	−0.122	**1**				
Zds	0.660	0.842	0.005	**1**			
Lcy-b	−0.233	−0.575	−0.222	−0.113	**1**		
Hy-b	0.774	0.725	−0.321	**0.927**[a]	0.147	**1**	
Zep	0.517	0.688	0.078	**0.971**[a]	0.096	**0.918**[a]	**1**

[a] Significant correlation ($p < 0.05$).

Table 6. Pearson Correlations between the 10 Carotenoid Variables and the Transcript levels of 7 Carotenoid Biosynthetic Genes in February

	Dxs	*Psy*	*Pds*	*Zds*	*Lcy-b*	*Hy-b*	*Zep*
total carotenoid	0.654	**0.837**[a]	−0.136	0.412	−0.888	0.261	0.184
phytoene	−0.171	0.330	0.615	−0.007	−0.899	−0.352	−0.142
phytofluene	−0.408	0.101	0.733	−0.179	−0.777	−0.526	−0.270
ζ-carotene	**−0.907**[a]	**−0.975**[a]	0.331	−0.761	0.550	−0.712	−0.587
lycopene	−0.004	0.484	0.528	0.130	−0.945	−0.206	−0.027
β-carotene	−0.941	−0.612	0.792	−0.607	−0.107	**−0.821**[a]	−0.530
β-cryptoxanthin	**0.839**[a]	0.613	−0.578	0.782	0.259	**0.956**[a]	0.768
zeaxanthin	0.808	0.307	**−0.970**[a]	0.237	0.216	0.541	0.165
cis-antheraxanthin	**0.947**[a]	0.590	**−0.873**[a]	0.404	−0.109	0.610	0.267
cis-violaxanthin	0.665	0.237	−0.747	0.464	0.571	0.761	0.503

[a] Significant correlation ($p < 0.05$).

in juice sacs of Cara Cara navel orange and the allelic variability of *Lcy-b* gene, we isolated *Lcy-b* alleles from Cara Cara navel, Shamouti, and Sanguinelli oranges; PCR amplifications were performed with genomic DNA from these three varieties as template. After purification, PCR products were cloned into the TOPO Cloning vector, and five clones per genotype were sequenced [*Lcy-b* is a single copy gene in the *Citrus* genome according to Fanciullino et al. (*37*)]. The *Lcy-b* clones were 1515 bp in length. These genomic sequences were compared to *Lcy-b* cDNA sequences, and no introns were found. Moreover, two alleles were isolated from each genotype. The genomic sequences of *Lcyb1* and *Lcyb2* from Cara Cara navel orange were different in 14 nucleotides, resulting in 3 amino acid changes. These two alleles (*Lcyb1* and *Lcyb2*) were the same alleles in Sanguinelli and Shamouti oranges (data not shown) and in Bonanza orange (GenBank accession no. DQ496221 and DQ496222).

DISCUSSION

Relationships between Carotenoid Composition of the Four Oranges and Steps of the Carotenoid Biosynthetic Pathway. One objective of the current work was to link the variations in carotenoid composition of the four oranges to the steps of the carotenoid biosynthetic pathway that may be involved in this differential carotenoid accumulation.

At a late developmental stage in February (282 dpa) Shamouti and Sanguinelli oranges were different from the two other oranges by higher proportions in β-cryptoxanthin, zeaxanthin, *cis*-antheraxanthin, and *cis*-violaxanthin (**Figures 3** and **4**). Therefore, these two genotypes presented common orange carotenoid profiles with β,β-xanthophyll accumulation in juices sacs (*6–10*). The differences in color parameter values between Shamouti and Sanguinelli were not related to carotenoid compositions (**Table 1**). Anthocyanins that are present in juice sacs of Sanguinelli should be responsible for the higher CIE *a** value (redness) compared to that of Shamouti juices (*1*). In addition, the transformations of β-carotene into β-cryptoxanthin and zeaxanthin catalyzed by HY-b and of zeaxanthin into antheraxanthin and violaxanthin by the action of ZEP should play a major role in β,β-xanthophyll accumulation (**Figure 1**). As well, degradation of *cis*-violaxanthin by the action of NCED should also explain the observed color phenotypes (**Figure 1**). Cara Cara orange was separated from the other oranges by high percentages of phytoene and lycopene (**Figures 3** and **4**). As was already shown in Cara Cara navel orange juice sacs (*11*), proportions of carotenes were higher than those in common oranges. Phytoene and lycopene accumulations suggested the importance of the first step of the carotenoid biosynthetic pathway catalyzed by PSY and also the importance of the cyclization of lycopene under the action of LCY-b. Huang pi Chen orange, which was

characterized for the first time, was differentiated from the other genotypes by low total carotenoid contents (**Figures 3** and **4**), which should be responsible for a low value for *a** (**Table 1**). The observed phenotype suggests that the first step of the carotenoid biosynthetic pathway catalyzed by PSY and the *cis*-violaxanthin degradation to produce ABA are key steps. The expression of the genes controlling these steps could be regulated at either transcriptional, post-transcriptional, or translational levels. In the following paragraphs we focus our discussion on the transcriptional regulation.

Regulation of Biosynthetic Genes at the Transcriptional Level and Relationships with Carotenoid Composition in Oranges. In this study, we attempted to evaluate the roles of the transcription level of seven genes in the carotenoid composition of four orange varieties. The coordination of the MEP and carotenoid pathways was also investigated.

Total carotenoid content in the four oranges was in agreement with the expression levels of *Dxs* and *Psy* at 282 dpa. Moreover, transcript levels of *Dxs* and *Psy* were positively correlated (**Table 5**). These findings suggest that in juice sacs, the expression of *Dxs* and *Psy* genes is coordinated, and this plays an important role in carotenoid accumulation. These results are in agreement with those of Lois et al. (*15*) on tomato (*Lycopersicon esculentum*) fruits, demonstrating that coordinated expression of these two genes controlled carotenoid synthesis. In *Citrus* fruits, the expression patterns of *Dxs* in juice sacs and flavedo are different because *Dxs* mRNA levels in the peel decrease with the degreening of fruits (*16*). A possible explanation for this difference could be a competition for GGPP between carotenoid and chlorophyll biosynthetic pathways in flavedo. The decrease in *Dxs* transcripts and the increase in *Psy* transcripts would allow a prevalure of carotenoid synthesis over chlorophyll. In juice sacs, very low amounts of chlorophylls are synthesized. This fact can explain the coordinated expression of *Dxs* and *Psy* genes. It is worth mentioning that in juice sacs of Huang pi Chen orange *Dxs* and *Psy* expression was relatively lower than in the other varieties, although this observation by itself does not appear to explain the practical absence of carotenoids is this variety. The carotenoid cleavage dioxygenase genes would not play an important role because the percentage of *cis*-violaxanthin in Huang pi Chen juices was high (20.6%) (*18*). However, ABA levels in this variety should be determined to confirm this hypothesis.

In juice sacs of Shamouti and Sanguinelli oranges, the accumulation of β,β-xanthophylls was associated with the increase in expression of carotenoid biosynthetic genes and, especially, with the up-regulation of the *Hy-b* gene. Indeed, high transcript levels of *Hy-b* gene at 282 dpa were correlated with high percentages of β-cryptoxanthin. The *Hy-b* transcripts increased 9-fold in Shamouti orange and 10-fold in Sanguinelli orange from 104 to 282 dpa. According to Kato et al. (*6*), the β,β-xanthophyll accumulation in juice sacs of Satsuma mandarin and Valencia orange was due to a simultaneous increase in the expression of genes *CitPsy*, *CitPds*, *CitZds*, *CitLcyb*, *CitHyb*, and *CitZep*. Our results are in agreement with those of Kato et al. (*6*) for Shamouti and Sanguinelli cultivars, which are characterized by the highest percentages of β,β-xanthophylls.

Surprinsingly, in Cara Cara navel orange, no significant difference was found in the transcript levels of *Pds*, *Zds*, and *Lcy-b* genes at 282 dpa when compared to Shamouti and Sanguinelli oranges. It was expected that the accumulation of carotenes could be related to down-regulation of the expression of these genes and especially by a down-regulation of *Lcy-b* expression. In addition, the Cara Cara navel orange phenotype could not be explained by mutations in the coding sequence of the *Lcy-b* gene. Indeed, the two alleles isolated from this genotype were also those of other oranges that did not accumulate lycopene. The large amount of phytoene in juice sacs of Cara Cara navel orange was not due to a higher expression of *Dxs* and *Psy* genes when compared to Shamouti and Sanguinelli oranges. These results are in agreement with those of Tao et al. (*38*). These authors showed that in juice sacs of Cara Cara navel and Washington oranges, no remarkable difference for *Psy* expression was observed, whereas phytoene exclusively accumulated in Cara Cara. Other regulation mechanisms should be involved such as post-transcriptional regulations, catalytic activity of enzyme, or plastid import of enzyme. According to Inoue et al. (*39*), LCY-b was synthesized as a precursor of about 60 kDa, targeted both to the soluble and to the membrane fractions of plastids, and was processed into a mature form of 45 kDa. These mechanisms could play a role in enzyme activation. Another explanation could be that the turnover of the carotenogenic enzymes would depend on the orange varieties, leading to contrasting activities of the enzymes and differential compound accumulation. The two enzymes (PDS and ZDS) needed plastoquinones and a plastid terminal oxidase as electron acceptors to catalyze desaturation reactions (*12*). Therefore, Cara Cara navel orange phenotype could also be explained by the lack of these cofactors. A similar situation has been found in Pinalate, a navel orange mutant that produces yellow mature fruits (*40*). Pinalate flavedo was characterized by an unusual accumulation of linear carotenes (phytoene, phytofluene, and ζ-carotene) and lower ABA contents than in the corresponding tissues of the parental variety Navelate. The authors suggested that an alteration in ζ-carotene desaturase or in ζ-carotene desaturase-associated factors is responsible for the Pinalate phenotype.

In conclusion, the results presented in this work revealed a general pattern of transcript change in juice sacs of citrus fruit, characterized by an apparent coordination of *Dxs* and *Psy* expression and a general increase in mRNA levels of carotenoid biosynthetic genes. These transcript changes correlated well with β,β-xanthophyll accumulation, the normal carotenoid set, observed in Shamouti and Sanguinelli oranges. The data also suggest that the preferential accumulation of linear carotenes in Cara Cara navel and the practical absence of carotenoids in Huang pi Chen oranges were not predominantly due to changes in the regulation of carotenoid biosynthetic genes at the transcriptional level.

ABBREVIATIONS USED

GGPP, geranylgeranyl diphosphate; HY-b, β-carotene hydroxylase; HY-e, ϵ-carotene hydroxylase; LCY-b, lycopene β-cyclase; LCY-e, lycopene ϵ-cyclase; MEP pathway, methylerythritol phosphate pathway; PDS, phytoene desaturase; PSY, phytoene synthase; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeats; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase.

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