JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Regulation of Color Break in Citrus Fruits. Changes in Pigment Profiling and Gene Expression Induced by Gibberellins and Nitrate, Two Ripening Retardants

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Citrus clementina fruits were repeatedly treated on-tree from mature green until breaker stages with either nitrate or gibberellin, two retardants of external ripening. The natural color break was characterized by a reduction in chlorophyll concentration, a decrease in β,ϵ-carotenoids, β-carotene, neoxanthin, and all-*E*-violaxanthin, and an increase in β,β-xanthophylls [mainly (*9Z*)-violaxanthin and β-cryptoxanthin]. The two retardants delayed both chlorophyll depletion and total carotenoid accumulation and in addition altered carotenoid composition. Treated fruits maintained longer the typical carotenoid composition of green fruits and reduced β,β-xanthophyll accumulation. Natural degreening was accompanied by a marked decrease in transcript levels of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and geranylgeranyl reductase (CHL P) while, conversely, pheophorbide a oxygenase (PaO) and phytoene synthase (PSY) gene expression increased. Gibberellin and nitrate delayed the reduction of DXS expression and the induction of PaO and PSY transcript accumulation, while no differences in CHL P were observed. The data indicate that both ripening retardants repressed natural PaO and PSY expression, suggesting a mechanistic basis for the elevated levels of chlorophyll and lower carotenoid concentration resulting from the gibberellin and nitrogen treatments and the consequent color break delay in citrus fruit peels.

KEYWORDS: Carotenoids; chlorophyll; *Citrus clementina*; gene expression; gibberellin; nitrate; ripening

INTRODUCTION

Citrus fruits are hesperidium berries, which differ from "true berries", such as tomato or grape, in having a leathery peel surrounding the edible fruit portion. The peel consists of an outer colored exocarp, called the flavedo, and an inner white spongy mesocarp, or albedo (*1*). Fruit growth in citrus species spans 6−12 months and follows a sigmoidal pattern that may be divided into three clear-cut stages (*2*): a first phase including cell division that results in slow growth (phase I); a rapid growth period (phase II) during which the fruit experiences a large increase in size involving cell enlargement and water accumulation; and finally, in phase III, growth is essentially arrested and fruits undergo nonclimateric ripening, including a characteristic color change. This process is of particular economical importance since the external color of citrus fruits is a critical quality parameter for the fresh market. The external color change results from the differentiation of chloroplasts to chromoplasts, a change that in citrus is influenced by environmental conditions, nutrient availability, and hormones (*3*). In general, internal ripening in citrus is coincident with external ripening, although there are exceptions indicating that these processes are regulated by separate control mechanisms. Despite its importance, the regulation of external color change in citrus is poorly understood and most of the knowledge gained in this area has resulted from observations generated through the exogenous application of various chemicals, such as hormones and nutrients. For example, postharvest treatments with ethylene have been used extensively since this hormone promotes the chloroplast to chromoplast transition and accelerates color break (*4*). However, the role of ethylene in the regulation of natural color break in nonclimateric citrus is still a matter of controversy since ethylene biosynthesis is minimal during phase III of fruit development when color changes take place (*5*). In contrast, gibberellin treatments are commonly used to delay color break and flavedo senescence in several citrus species (*6*). In addition, a decrease in nitrogen content and an increase in sucrose levels have been shown to promote color changes in the flavedo of citrus fruits (*3*, *7*). The color break in citrus fruits, therefore, appears to be under the control of a network of regulatory metabolic signals, including ripening inducers such as ethylene

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10.1021/jf0606712 CCC: $33.50
Published on Web 06/06/2006

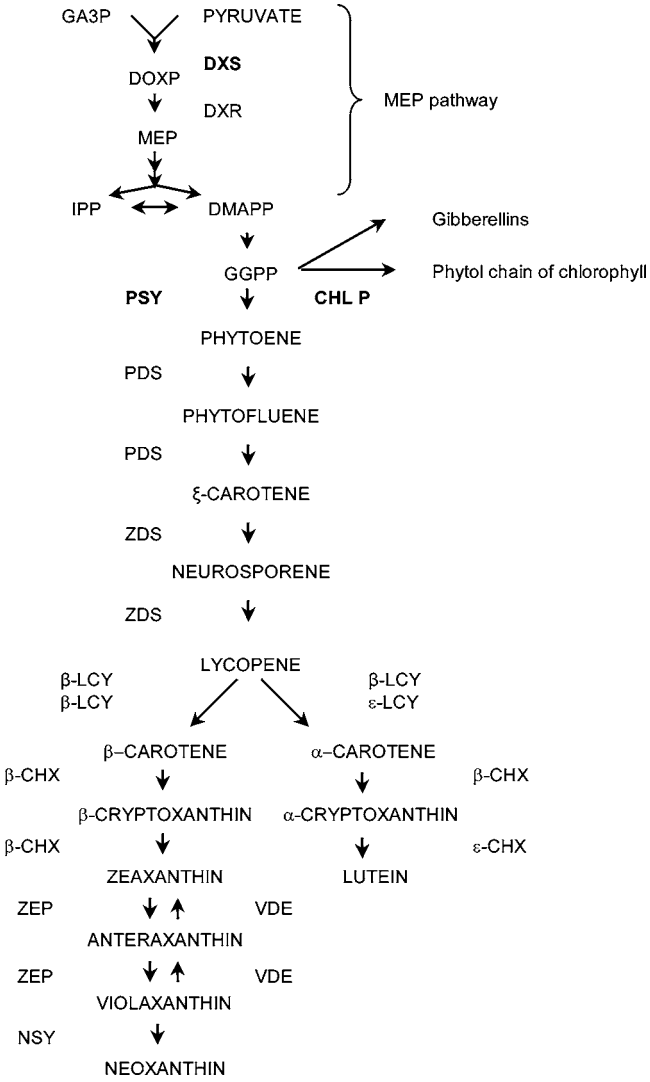

Figure 1. Overview of the plastidial isoprenoid biosynthesis pathway. Several steps are omitted for simplification. GA3P, glyceraldehyde 3-phosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; PDS, phytoene desaturase; ZDS, ξ-carotene desaturase; β-LCY, β-lycopene cyclase; ε-LCY, ε-lycopene cyclase; β-CHX, β-carotene hydroxylase; ε-CHX, ε-carotene hydroxylase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase; and NSY, neoxanthin synthase.

and sucrose and ripening retardants, including gibberellins and nitrogen.

Citrus peel is a complex source of carotenoids. This tissue contains the greatest diversity of carotenoids of any fruit studied to date and their specific accumulation patterns are responsible for the broad range of colors exhibited by citrus fruits (*8*). Although the characteristic color of typical citrus varieties is mainly provided by the accumulation of (9*Z*)-violaxanthin and β-cryptoxanthin, there are also citrus-specific apocarotenoids such as β-citraurin and β-citraurinene that provide an attractive coloration and whose biosynthethic basis remains unknown (*9*). **Figure 1** summarizes the main steps of the carotenoid biosynthetic pathway in higher plants. Cytoplasmic and mitochondrial isoprenoids are thought to be formed from acetyl-coA via mevalonate, while plastidial isoprenoids such as carotenoids, gibberellins, tocopherol, and the phytol chain of chlorophylls are formed from the 2-methyl-erythritol-phosphate (MEP) pathway (*10*). Four molecules of isopentenyl diphosphate form the C_{20}-intermediate geranylgeranyl diphosphate (GGPP) that can be used to synthesize gibberellins, phytyl diphosphate via geranylgeranyl reductase (CHL P) and phytoene via phytoene synthase (PSY), the first committed step in carotenogenesis. In this pathway, cyclization of lycopene, the first branching point, leads to β-carotene and its xanthophyll derivatives or to α-carotene and lutein (*11*).

While the mechanistic basis of color break in citrus has not yet been resolved, some studies have suggested factors that contribute to pigment turnover. For example, it has been shown that chlorophyllase, a constitutively expressed gene, does not increase during natural fruit development (*12*). In addition, the expression patterns of carotenoid biosynthetic genes and associated abundance of carotenoids during ripening have recently been reported (*13*, *14*). Other aspects of color break may be inferred from studies in other fruit species and developmental processes. Thus, research in tomato strongly suggested the involvement of other upstream genes, such as 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (*15*), on the regulation of carotenoid accumulation. Studies on leaf senescence have also revealed the potential participation of CHL P and pheophorbide a oxygenase (PaO) in chlorophyll breakdown (*16*, *17*). However, overall, little is known about the molecular basis of color break in citrus and its regulation.

The aim of this current study was to analyze the patterns of pigment accumulation in citrus flavedo and the expression of selected genes that are affected by treatments with gibberellin and nitrate to provide insight in the regulation of color change. *Citrus clementina*, a high-quality cultivar of major commercial importance in the citrus industry of the Mediterranean basin, was used.

MATERIALS AND METHODS

Plant Material and Fruit Treatments. Parthenocarpic fruits of *C. clementina* (cv. Clementina de Nules) mandarin were harvested from adult trees grown in a homogeneous orchard located at the Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain) under normal culture practices. Sampling dates were August 2 [123 DPA (days postanthesis), phase II, immature green (IG) stage], October 7 [189 DPA, phase II, mature green (MG)], October 25 [207 DPA, prebreaker (PB) stage], and November 18, 2004 [231 DPA, breaker stage (B), beginning of phase III]. Individual labeled fruits were treated on-tree with either 25 mM potassium nitrate + 25 mM calcium nitrate or 60 mg L^{-1} gibberellin A_3 (GA_3) (Sigma, Madrid, Spain). In each treatment, fruits on four adult trees were sprayed every 3 days from 189 DPA to 231 DPA. All trees were divided into four parts, north, south, east, and west, according to the spatial orientation, and each treatment was applied once to the four orientations. After color index determination (see below), flavedo tissue was frozen under liquid nitrogen, ground to a fine powder, and stored at −80 °C until pigment analysis or RNA extraction.

Color Index Determination. The *L* (0−100, black to white), *a* (± yellow/blue), and *b* (± red/green) Hunter lab parameters of the color system were measured with a Minolta CR-200 chromameter. The values presented are the results of the 1000 *a*/*Lb* transformation that results in negative and positive values for the green and orange colors, respectively, in citrus fruit. In this transformation, the zero value coincides with the midpoint of the color break period (*18*). The color index was determined after three determinations around the equatorial plane of the fruit. Twenty fruits per sample were used.

Pigment Extraction and Quantification. Chlorophyll and carotenoids were extracted as previously described by Rodrigo et al. (*19*). Chlorophylls a and b and total (a + b) contents were determined following the Smith and Benitez (*20*) equations after measuring the absorbances at 644 and 662 nm. The pigment ethereal solution was then dried and saponified using 10% methanolic:KOH solution. Carotenoids were subsequently re-extracted with diethyl ether until the hypophase was

Table 1. Oligonucleotides Used as Primers for RT-PCR[a]

gene	primer	primer amount[b] (μL)	amplicon size (pb)
DXS-forward	5′-CGTGTTTTCAACACACCTGACG-3′	3.6	120
DXS-reverse	5′-AAGCCCCGAAGTCTTCCTCAT-3′	1.2	
CHL P-forward	5′-GCGTGATTAACGGGTTAT-3′	3.6	353
CHL P-reverse	5′-CACTGTGCCTGTACCA-3′	3.6	
PSY-forward	5′-GGTCGTCCATTTGATATGCTTG-3′	1.2	111
PSY-reverse	5′-CCTAAGGTCCATCCTCATTCCT-3′	0.2	
PaO-forward	5′-GCGACAAATGACGGTAAAAAGC-3′	3.6	103
PaO-reverse	5′-CGTTTCTTCATCTGATGCTGCA-3′	3.6	

[a] Oligonucleotides were designed using Primer Express Software (Applied Biosystems). [b] Optimized amounts of a 5 μM oligonucleotide solution.

colorless. An aliquot of this extract was used for total carotenoid content quantification by measuring the absorbance at 450 nm and using the extinction coefficient of β-carotene, $E^{1\%} = 2500$ (*21*). Extracts were then dried under N_2 and kept at −20 °C until high-performance liquid chromatography (HPLC) analysis. All operations were carried out on ice and under dim light to prevent photodegradation, isomerizations, and structural changes of carotenoids.

HPLC Analysis of Carotenoids. Dried carotenoid extracts were dissolved in methanol:acetone (2:1, v/v). Chromatography was carried out with a Waters liquid chromatography system equipped with a 600E pump, a model 996 photodiode array detector, and the Millenium Chromatography manager software version 2.0 (Waters, Barcelona, Spain). A C_{30} carotenoid column (250 mm × 4.0 mm, 5 μm) coupled to a C_{30} guard column (20 mm × 4.6 mm, 5 μm) (YMC EuropeGMBH, Schermbeck, Germany) was used for analysis. Carotenoid pigments were analyzed by HPLC using the ternary gradient elution with methanol, water, and methyl *tert*-butyl ether reported in a previous study (*22*). The photodiode array detector was set to scan from 250 to 540 nm throughout the whole elution profile. The area of each peak was calculated as a proportion of the total area of carotenoid peaks in a Maxplot chromatogram, which plots each carotenoid peak at its corresponding maximum absorbance wavelength. Each sample was extracted and analyzed at least twice. β-Carotene, α-carotene, and lutein from Sigma-Aldrich (Madrid, Spain) and β-cryptoxanthin and zeaxanthin from Extrasynthese (Lyon, France) were used as standards.

RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR). The total RNA was isolated from frozen flavedo using the RNeasy Plant Mini Kit (Qiagen) and treated with RNase-free DNase (Qiagen) through column purification according to the manufacturer's instructions. UV absorption spectrophotometry and gel electrophoresis were performed to test RNA quality as described by Sambrook et al. (*23*).

Quantitative RT-PCR was performed with a LightCycler 2.0 Instrument (Roche) equipped with LightCycler Software version 4.0. One-step RT-PCR was carried out with 25 ng of total RNA adding 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 optimized amounts of gene-specific primers (**Table 1**) in a total volume of 10 μL. Incubations were carried out at 48 °C for 30 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 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 assesed by post-amplification 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 a standard curve. For normalization of mRNA abundance, two different methods were utilized. In initial experiments, relative mRNA levels were normalized to the 18S rRNA abundance. However, this method introduced elevated variability to the measurements and rendered irreproducible results since very high sample dilutions were required to fit the large pool of the 18S rRNA within the dynamic range of the RT-PCR instrument, a technical limitation previously reported (*24*, *25*). Satisfactory reproducibility of the data was obtained after normalization to total RNA amounts accurately quantified with the RNA-specific fluorescent dye RiboGreen (Molecular Probes, Eugene, OR; *24*, *25*). Induction values of one-fold were arbitrarily assigned to the 123 DPA sample. Each sample was analyzed in triplicate, and analysis of variance tests were performed to assess the statistical significance of differences in gene expression between treated and control fruits.

The sequences of the primers used for the RT-PCR are shown in **Table 1**. The DXS primers were designed based on the EST CX295610 sequence (77% amino acid identity with *Catharanthus roseus* DXS, AJ 011840) isolated from a cDNA library of leaves from *C. clementina* (*26*). Similarly, CHL P and PaO primers were based on the EST DY275174 sequence (75% amino acid identity with *Medicago truncatula* CHL P, AY960125) and EST DY261492 sequence (81% amino acid identity with *Lycopersicon esculentum* PaO, AF321984), respectively, both from a normalized full length cDNA library of several vegetative and reproductive tissues of *C. clementina* (to be published, *27*). PSY primers design was based on the sequence of *Citrus unshiu* PSY (AF220218, *28*).

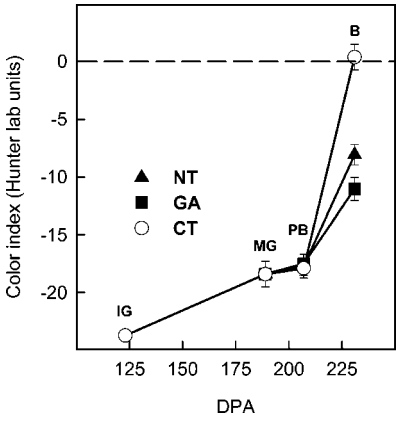

Figure 2. Changes in color index in the flavedo of *C. clementina*. Fruit color is expressed as 1000 *a*/*Lb* (Hunter Lab units) (*18*). In this formula, positive values indicate orange color and negative values indicate green color, while zero corresponds to the B. The data are means ± SD of 20 replicates. CT, control; NT, nitrate; and GA, gibberellin. Treatments started at 189 DPA.

RESULTS

Effect of Nitrate and Gibberellin on Color Index, Chlorophylls, and Total Carotenoids. The color index in flavedo of *C. clementina* fruits was measured at four developmental stages: IG (123 DPA, during the cell enlargement phase), MG (189 DPA, when fruits reached their full size), PB (207 DPA), and B (231 DPA, at color break, when color index equals 0). In control fruits, the color index increased 5.8 Hunter lab units (−23.7 to −17.9) between the IG and the PB stages. Then, color change accelerated from PB to B (207−231 DPA) and increased 18 Hunter lab units (−17.9 to +0.38) in 24 days (**Figure 2**). In contrast, gibberellin- and nitrate-treated flavedo tissues remained green, in the negative color index range (around −10) at 231 DPA (**Figure 2**).

The total chlorophyll concentration did not change from IG to MG (123−189 DPA), whereas levels were dramatically reduced at PB and B stages. At color break, the total chlorophyll content was very low (32.7 μg/g FW, **Figure 3A**), and at this stage, chlorophyll was higher in nitrate- and gibberellin-treated fruits that had similar levels (67.2 and 66.2 μg/g FW, respectively, **Figure 3A**). This difference was found for both chlorophylls a and b (data not shown).

The total carotenoid content in control flavedo showed a transient increase at 189 DPA (MG stage), followed by a decrease (207 DPA, PB stage) prior to the accumulation period

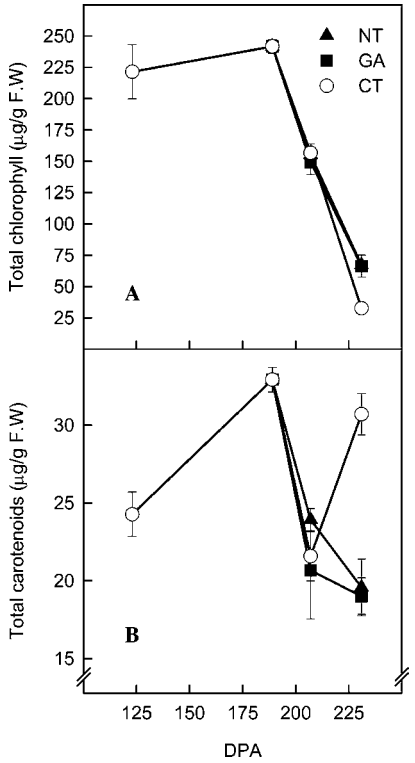

Figure 3. (**A**) Total chlorophyll contents and (**B**) total carotenoid content (expressed as β-carotene equivalents) in the flavedo of *C. clementina*. Data are means ± SD of three measurements. CT, control; NT, nitrate; and GA, gibberellin. Treatments started at 189 DPA.

at 231 DPA (B stage). The decrease at 207 DPA was consistently detected in controls and in all treatments, although the carotenoid concentration rise observed in control fruit at 231 DPA did not occur in either nitrate- or gibberellin-treated fruits (**Figure 3B**).

Effect of Nitrate and Gibberellin on Carotenoid Composition. The carotenoid composition of the flavedo of *C. clementina* was analyzed by HPLC during normal development and ripening (IG, MG, PB, and B stages) and after nitrate and gibberellin treatments (**Figure 4**). A total of 11 carotenoids were tentatively identified through comparison of the spectra, the chromatographic retention times, and the numerical notations (% III/II) with those of authentic standards when available or with data reported in the literature (**Table 2**). Phytofluene and α-cryptoxanthin were at trace levels (0.3−0.5 and 0.4−1.3%, respectively), and although identification was possible, quantification was not deemed to be reliable. Zeaxanthin was detected at consistently low levels and was not affected by the treatments. In addition, low levels of unidentified carotenoids were found in all samples (data not shown). Other carotenoids such as ζ-carotene, cryptoflavin, and citraurin were not found in the extracts, although some of them, for example, citraurin, have been reported to accumulate at detectable levels only in ripened citrus flavedo (*9*).

In fruits that contained high amounts of chlorophyll (123−189 DPA, IG and MG stages), lutein was the most abundant carotenoid (41 and 36% of the total content, **Figure 4**), suggesting that the α-carotene branch leading to lutein is an active part of the biosynthetic pathway in green fruits. Other over-represented carotenoids in green fruits were all-*E*-violaxanthin, neoxanthin, and α/β-carotene (**Figure 4**). Phytoene accumulated at very low levels, with a peak at the MG stage (189 DPA), before substantial changes in chlorophyll content and carotenoid composition (**Figures 3A** and **4**).

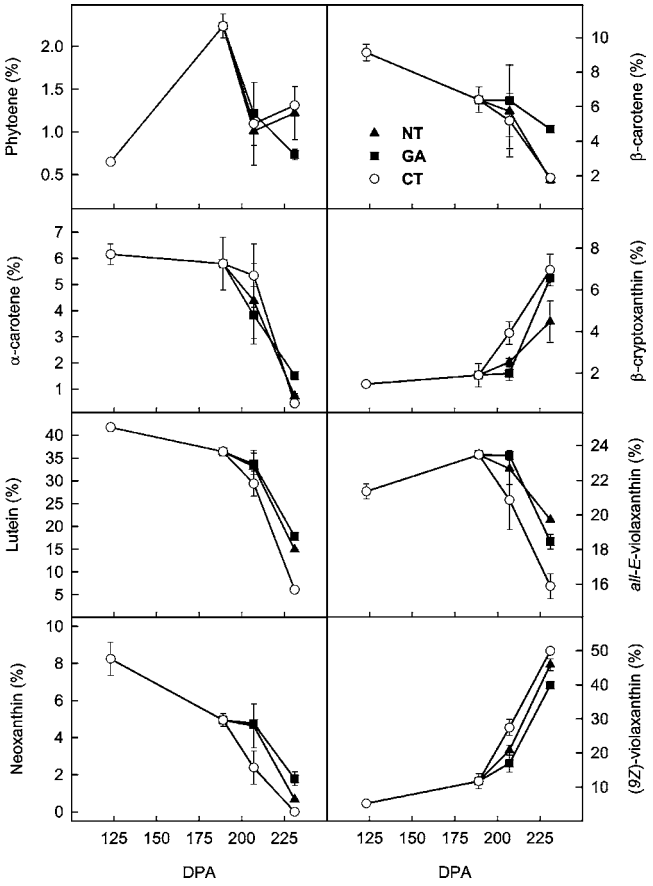

Figure 4. Relative levels of carotenoids in the flavedo of *C. clementina* expressed as percentage of the total based on Maxplot chromatograms. Data are means ± SD of three measurements. CT, control; NT, nitrate; and GA, gibberellin. Treatments started at 189 DPA.

Analysis of the carotenoid composition of fruits at the PB and B stages revealed a different accumulation pattern with a greater predominance of β,β-xanthophylls. In 24 days (from 207 to 231 DPA, PB to B), lutein and α-carotene contents decreased 80 and 90%, respectively, while (*9Z*)-violaxanthin increased 81% (**Figure 4**). Thus, in B fruits, (*9Z*)-violaxanthin was the most abundant carotenoid (50%) while lutein was present at very low levels (6%). It is also noteworthy that all-*E*-violaxanthin markedly decreased in B fruits. From MG to B stages, the β-carotene percentage decreased, an observation compatible with the fact that the amounts of β,β-xanthophylls such as β-cryptoxanthin (orange) and (*9Z*)-violaxanthin (yellow) increased in a substrate−product manner. Neoxanthin that represented almost 10% of the carotenoids at IG stage became undetectable at the B stage (**Figure 4**).

Nitrate and gibberellin treatments at 207 DPA clearly influenced the β-carotene branch (**Figure 4**) and had similar effects, inducing a decrease in the proportion of (*9Z*)-violaxanthin and β-cryptoxanthin and maintaining relatively high neoxanthin levels. Later, when differences in color index, chlorophyll, and total carotenoid levels were noticeable (231 DPA), treated fruits showed higher percentages of the typical carotenoids of green fruits. Thus, gibberellin-treated fruits had 2.9-, 3.3-, 2.5-, and 1.2-fold more lutein, α-carotene, β-carotene, and all-*E*-violaxanthin, respectively, than controls (**Figure 4**), and neoxanthin, which was absent in controls, was still present in treated fruits (1.8%). However, on the contrary, phytoene and (*9Z*)-violaxanthin were less abundant. Nitrate and gibberellin promoted major changes in the carotenoid composition of treated flavedo, resulting in a profile that resembled that of green fruits.

Table 2. Chromatographic and Spectroscopic Characteristics of the Most Relevant Carotenoids in the Flavedo of *C. clementina* cv. Clementina de Nules

observed				literature		
t_R (min)	carotenoid[a]	λ_{max} (nm)	peak ratio[b]	λ_{max} (nm)	peak ratio[b]	ref
14.75	all-*E*-violaxanthin	411, 435, 463	92	416, 438, 467	87	*41*
15.47	neoxanthin	411, 435, 463	90	412, 434, 464	85	*41*
19.97	(9*Z*)-violaxanthin	cis326, 410, 434, 463	95	cis326, 416, 440, 465	98	*41*
23.25	lutein[a]	418, 444, 472	65	421, 445, 474	60	*41*
25.77	zeaxanthin[a]	430, 450, 478	35	428, 450, 478	26	*41*
27.76	α-cryptoxanthin	423, 444, 479	30	428, 450, 478	27	*41*
29.02	phytoene	273, 284, 300	10	276, 286, 297	10	*41*
31.25	phytofluene	331, 346, 365	91	331, 348, 367	90	*41*
33.48	β-cryptoxanthin[a]	423, 448, 475	30	428, 450, 478	27	*41*
38.64	α-carotene[a]	420, 450, 473	62	422, 445, 473	55	*42*
41.79	β-carotene[a]	426, 451, 478	31	425, 450, 477	25	*41*

[a] Identified through comparison with authentic standards. [b] Peak ratio is % III/II carotenoids (*41*).

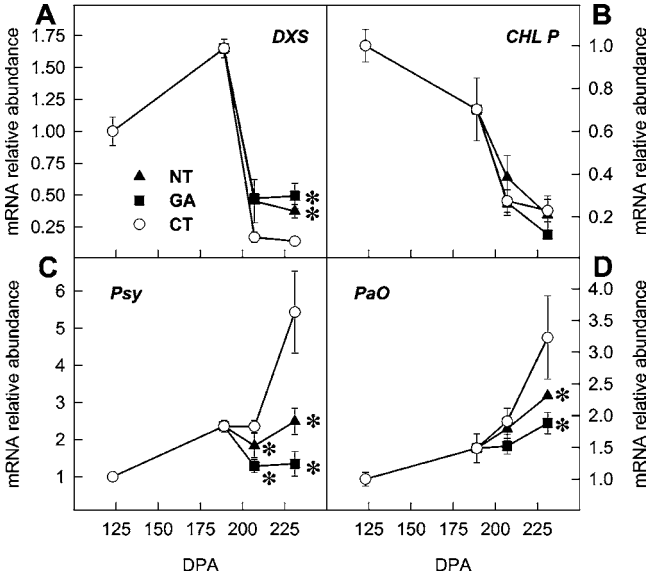

Figure 5. Expression profiles in the flavedo of *C. clementina* of DXS (**A**), CHL P (**B**), PSY, (**C**) and PaO; (**D**) genes were determined by real-time RT-PCR. An expression value of 1 was arbitrarily assigned to the 123 DPA sample. Data are means ± SD of three measurements. CT, control; NT, nitrate; and GA, gibberellin. Treatments started at 189 DPA. The asterisks indicate statistically significant differences of gene expression between control and treated fruits at the level of $P \leq 0.05$ (for PaO expression in NT-treated flavedo at 231 DPA, $P = 0.055$).

Thus, both ripening retardants clearly delayed lutein and all-*E*-violaxanthin reduction. The only differences between the two treatments were minimal; for example, nitrogen, but not gibberellin, had apparently a minor or no effect on phytoene and α/β-carotene levels, while nitrogen was more effective in reducing β-cryptoxanthin (**Figure 4**).

Effect of Nitrate and Gibberellin on Gene Expression. The expression levels of genes associated with potential regulatory enzymes of pigment biosynthesis or degradation were quantified by RT-PCR and compared with the effects induced by nitrate and gibberellins (**Figure 5**). These genes included *DXS* (*15*), *PSY* (the first committed step in the carotenoid pathway), *CHL P* (geranygeranyl reductase, which reduces GGPP to phytyl diphosphate), and *PaO* (which opens the porphyrin macrocycle of pheophorbide, *17*).

DXS expression showed a transient increase at MG stage (189 DPA), strongly decreased at PB stage, and remained low later on. Nitrate and gibberellin treatments maintained moderate *DXS* transcript levels during PB and B stages (2.5-fold, **Figure 5A**). The expression of *CHL P* was severely reduced along the studied period, and none of the treatments changed the natural expression of this gene (**Figure 5B**). In contrast, *PSY* transcript levels increased more than 5-fold from IG to B stages. Nitrate and gibberellin treatments markedly affected *PSY*, lowering expression levels. These differences were statistically significant ($P \leq 0.05$) at 207 and 231 DPA (**Figure 5C**). *PaO* was up-regulated during ripening and had the highest transcription levels at B stage. Nitrate- and gibberellin-treated flavedos showed lower *PaO* expression than the control tissues (**Figure 5D**).

DISCUSSION

In citrus as in many other fruits, color change, the result of chloroplast to chromoplast transition, involves chlorophyll degradation and carotenoid accumulation. These processes are thought to be under environmental, nutritional, and hormonal control. While the influence of environmental conditions on chromoplast transformation has been deduced from exposing plants to different environmental regimes, for example, low temperatures (*29*), the effects of nutrients and hormones are typically assessed by external chemical application. Thus, it has been demonstrated that sucrose accelerates color change in citrus fruit flavedo, while nitrogen delays chromoplast formation (*3*, *7*). It has also been shown that ethylene and gibberellin have opposite effects on regulating chorophyll degradation in flavedo (*12*). While much is known about the role of ethylene in regulating fruit ripening (*12*, *30*−*33*), very little attention has been paid to the specific mechanisms that delay color change. In this current study, two ripening retardants of citrus peel, nitrate (nutritional) and gibberellin (hormonal), have been selected to study their effects on the metabolic profiling and gene expression associated with the pigments responsible for color change.

During natural external ripening, chlorophylls (**Figure 3A**) were strongly reduced and carotenoid composition was substantially altered (**Figure 4**). Chlorophyll concentration showed a continuous decrease starting at the PB stage (**Figure 3A**), whereas total carotenoid concentration exhibited a transient increase at 189 DPA (MG stage), followed by a decrease at 207 DPA (PB stage) just before carotenoid accumulation (**Figure 3B**). The decrease prior to the B stage, which has also been previously observed in the flavedo of other mandarin and orange cultivars (*14*, *33*, *34*), is typical of fruits that synthesize large amounts of carotenoids during ripening (*8*). In these fruits, the total carotenoid content shows minimum levels at midseason, coincident with the disintegration of the granal system and the loss of chlorophyll. Thus, the observed carotenoid reduction

might be a consequence of the transition from chloroplasts to chromoplasts. The measurements presented in **Figure 4** revealed the individual changes implicated in the chloroplast to chromoplast transformation. Green fruit tissues were characterized by high levels of lutein, all-*E*-violaxanthin, α/*β*-carotene, and neoxanthin, whereas B fruits were enriched in (*9Z*)-violaxanthin and *β*-cryptoxanthin. At the PB stage, the spectrum of carotenoids underwent a shift from the α-carotene branch to the *β*,*β*-xanthophyll branch (**Figure 4**), and later, at the B stage, the predominance of the *β*,*β*-branch was clear. In comparison with naturally ripened fruits, the flavedo of nitrogen- and gibberellin-treated fruits contained higher levels of chlorophylls and had a carotenoid profile that was typical of green tissues. The combined changes in pigment levels explain the delay in the changes of color index that was induced by nitrogen and gibberellin and suggests that the two retardants delay both the chlorophyll turnover and the differential flux through the bifurcated carotenoid pathway that occurs during normal ripening. It is possible that the retardants exert differential effects on specific steps of the carotenoid pathways since, for example, the data indicated that gibberellin was more effective than nitrate in maintaining higher levels of both α- and *β*-carotene. However, it should be noted that this difference might reflect the differential potency of the retardants at the concentrations that were used in this study.

Previous studies of the regulation of color change in citrus flavedo have almost exclusively focused on the enzyme chlorophyllase. Jacob-Wilk et al. (*12*) concluded that gibberellins reduced chlorophyllase expression in ethylene-treated and nontreated citrus fruits, although the role of this constitutive gene in natural degreening remains to be proven. The expression of carotenoid biosynthetic genes during normal chromoplast transformation has also been recently reported (*13*, *14*). In this current study, the transcript levels of four potential regulatory steps of pigment biosynthesis were evaluated: DXS, a hypothetical control step of carotenoid biosynthesis in tomato fruit (*15*); PSY, the first committed step in the carotenoid pathway; CHL P, which catalyzes the reduction of GGPP to phytyl diphosphate; and PaO, which has been suggested to be a key regulator of chlorophyll catabolism (*17*). One of the initial observations of the present work, based on gene expression analysis and pigment profiling, is that biosynthesis of both chlorophylls and carotenoids might be coordinated. During natural ripening, the highest DXS expression levels at the MG stage coincided with a peak in total carotenoid and chlorophyll abundance, suggesting that precursors from the MEP pathway were used for chlorophyll and carotenoids biosynthesis. However, at the PB and B stages, DXS expression remained low, PSY expression increased, and CHL P transcript abundance decreased. This suggests that during ripening, the flux through the carotenoid biosynthetic pathway increased, while conversely metabolic flux leading to chlorophyll synthesis decreased. Expression of DXS in ripening tomato, on the contrary, was positively coordinated with that of PSY and coincided with carotenoid accumulation. These observations supported the suggestion that tomato DXS may be the first regulatory step in carotenoid biosynthesis (*15*). The results of this current study suggest that in citrus competition for GGPP between PSY and CHL P at the branching step is a pivotal element in controlling the differential rate of chlorophyll and carotenoid accumulation. Furthermore, it was shown that transformation of tomato with a PSY-1 cDNA resulted in a depletion of gibberellin levels because of the substantial redirection of GGPP into phytoene biosynthesis (*35*). Consistently with this hypothesis, the reduction in PSY expression levels detected in nitrate- and gibberellin-treated flavedos may explain the lower accumulation of total carotenoids induced by the retardants (**Figures 3B** and **5**). Similarly, it appears that chlorophyll degradation during natural ripening and chlorophyll retention after retardant treatments were mostly related to the modulation of PaO expression, since PaO transcript levels were higher and lower, respectively, while those of CHL P did not change after the treatments (**Figures 3A** and **5**). Moreover, PaO activity has been correlated with chlorophyll loss in barley leaves both during natural senescence and after hormonal treatments (*36*). The ability of nitrogen and gibberellin to delay color change has been known for a long time, although the mechanisms involved are poorly understood. In citrus, it is known that nitrogen depletion is required for color change (*3*, *7*) and it has also been suggested that low temperatures are essential for chromoplast formation (*29*), because nitrogen uptake and translocation are dramatically reduced under those conditions (*37*). In addition, a number of studies have correlated higher chlorophyll levels with elevated N content and vice versa (*38*, *39*). Similarly, there is a strong association between gibberellin and high chlorophyll concentration during several physiological processes. For example, gibberellin treatments are used commercially to delay color break in citrus (*4*, *6*) and gibberellin is known to be involved in delaying leaf senescence and associated chlorophyll retention (*40*).

In conclusion, we have shown that in the flavedo of citrus fruit, the transition from chloroplasts to chromoplasts results in reduced levels of chlorophyll and lutein and in elevated *β*,*β*-xanthophyll levels. The data indicate that the ripening retardants, nitrogen and gibberellin, operate via a reduction in chlorophyll depletion and the maintenance of *β*,*ϵ*-carotenoids, to the detriment of flux through the *β*,*β*-branch. In addition, the results suggest that both promoted similarly effects through an increase in DXS transcription and a reduction of PSY and PaO expression. The changes in gene transcript accumulation offer a plausible explanation for the color break delay and the differential pigment profiling observed in treated fruits.

ABBREVIATIONS USED

PaO, pheophorbide a oxygenase; DPA, days postanthesis; IG, immature green; MG, mature green; PB, prebreaker; B, breaker stage; GA_3, gibberellin A_3; GA3P, glyceraldehyde 3-phosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP, 2-C-methyl-D-erythritol 4-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; CHL P, geranylgeranyl reductase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, *ξ*-carotene desaturase; *β*-LCY, *β*-lycopene cyclase; *ϵ*-LCY, *ϵ*-lycopene cyclase; *β*-CHX, *β*-carotene hydroxylase; *ϵ*-CHX, *ϵ*-carotene hydroxylase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase.

ACKNOWLEDGMENT

The technical assistance of Isabel Sanchis and Amparo Beneyto is gratefully acknowledged. We thank Jocelyn Rose for critical comments and editing of the manuscript.

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Received for review March 9, 2006. Revised manuscript received May 5, 2006. Accepted May 5, 2006. This work was partially supported by INIA (RTA04-013, RTA03-106, and RTA05−247). E.A. was the recipient of a predoctoral fellowship from INIA.

JF0606712