JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Modification of Carotenoid Levels by Abscission Agents and Expression of Carotenoid Biosynthetic Genes in 'Valencia' Sweet Orange

Fernando Alferez,*[,†,§] Luis V. Pozo,[†] Russell R. Rouseff,[‡] and Jacqueline K. Burns[†]

[†]Horticultural Sciences Department and [‡]Department of Food Science and Human Nutrition, University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, Florida 33850-2299, United States

[§]Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), Apartado Postal 73, 46100 Burjassot, Valencia, Spain

ABSTRACT: The effect of 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP) and ethephon on peel color, flavedo carotenoid gene expression, and carotenoid accumulation was investigated in mature 'Valencia' orange (*Citrus sinensis* L. Osbeck) fruit flavedo at three maturation stages. Abscission agent application altered peel color. CMNP was more effective than ethephon in promoting green-to-red (*a*) and blue-to-yellow (*b*) color at the middle and late maturation stages and total carotenoid changes at all maturation stages. Altered flow of carotenoid precursors during maturation due to abscission agents was suggested by changes in phytoene desaturase (*Pds*) and ζ-carotene desaturase (*Zds*) gene expression. However, each abscission agent affected downstream expression differentially. Ethephon application increased β-carotene hydroxilase (*β-Chx*) transcript accumulation 12-fold as maturation advanced from the early to middle and late stages. CMNP markedly increased β- and ε-lycopene cyclase (*Lcy*) transcript accumulation 45- and 15-fold, respectively, at midmaturation. Patterns of carotenoid accumulation in flavedo were supported in part by gene expression changes. CMNP caused greater accumulation of total flavedo carotenoids at all maturation stages when compared with ethephon or controls. In general, CMNP treatment increased total red carotenoids more than ethephon or the control but decreased total yellow carotenoids at each maturation stage. In control fruit flavedo, total red carotenoids increased and yellow carotenoids decreased as maturation progressed. Trends in total red carotenoids during maturation were consistent with measured *a* values. Changes in carotenoid accumulation and expression patterns in flavedo suggest that regulation of carotenoid accumulation is under transcriptional, translational, and post-translational control.

KEYWORDS: *5-Chloro-3-methyl-4-nitro-1H-pyrazole, citrus, ethephon, Hunter Lab color space value, maturation, mechanical harvesting, peel and juice color*

INTRODUCTION

Citrus differs from most other tree fruit in that the fruit from several cultivars do not abscise easy and uniformly when mature. The pull force to remove the fruit is high unless abscission agents are employed. Due to increasing labor costs, the use of mechanical harvesting technologies to harvest processing oranges is increasing in Florida. Adoption of these technologies in Florida will be accelerated with registration and use of an abscission agent.[1] Several compounds have been tested in Florida as abscission agents, including methyl jasmonate,[2] coronatine,[3] metsulfuron methyl,[4] ethephon (2-chloroethylphosphonic acid), and CMNP (5-chloro-3-methyl-4-nitro-1*H*-pyrazole).[5] In particular, ethephon and CMNP are suitable abscission agent candidates. To be fully accepted by the citrus industry, the impact of these agents on fruit appearance and product quality must be determined when used throughout the harvest season. Previous research demonstrated that citrus peel color was influenced by abscission agents.[6] CMNP was more effective than ethephon in accelerating color change in fruit. Total carotenoids were higher in CMNP-treated fruit peel, especially when applied in the latter part of the harvest season. However, specific carotenoid changes and regulation at the transcriptional level brought about by both compounds are not known.

Citrus fruit are among the richest and most complex sources of carotenoids in plants. [7] Concentration and composition of carotenoids vary greatly among fruit of citrus cultivars and may depend upon factors such as cultural conditions and phytohormone treatment.[8,9] In citrus peel and juice sacs, carotenoid fluctuation during maturation is regulated by coordinated expression of genes of the carotenoid biosynthetic pathway (Figure 1).[10,11] As fruit mature, increased phytoene desaturase (*Pds*) and ζ-carotene desaturase (*Zds*) gene expression resulted in increased downstream carotenoid content. Color development characteristic of mature citrus fruit was associated with downregulation of ε-lycopene cyclase (*ε-Lcy*) and upregulation of β-lycopene cyclase (*β-Lcy*) gene expression and a shift from α-carotene and lutein production to β-carotene, β-cryptoxanthin, zeaxanthin, and violaxanthin production.[10,11] Lutein, the most abundant carotenoid in green tissues, and its precursor α-carotene are characteristic of chloroplastic tissues. Violaxanthin, the main carotenoid in orange fruit peel, accumulates during growth and development

Received: December 17, 2012
Revised: March 1, 2013
Accepted: March 2, 2013
Published: March 2, 2013

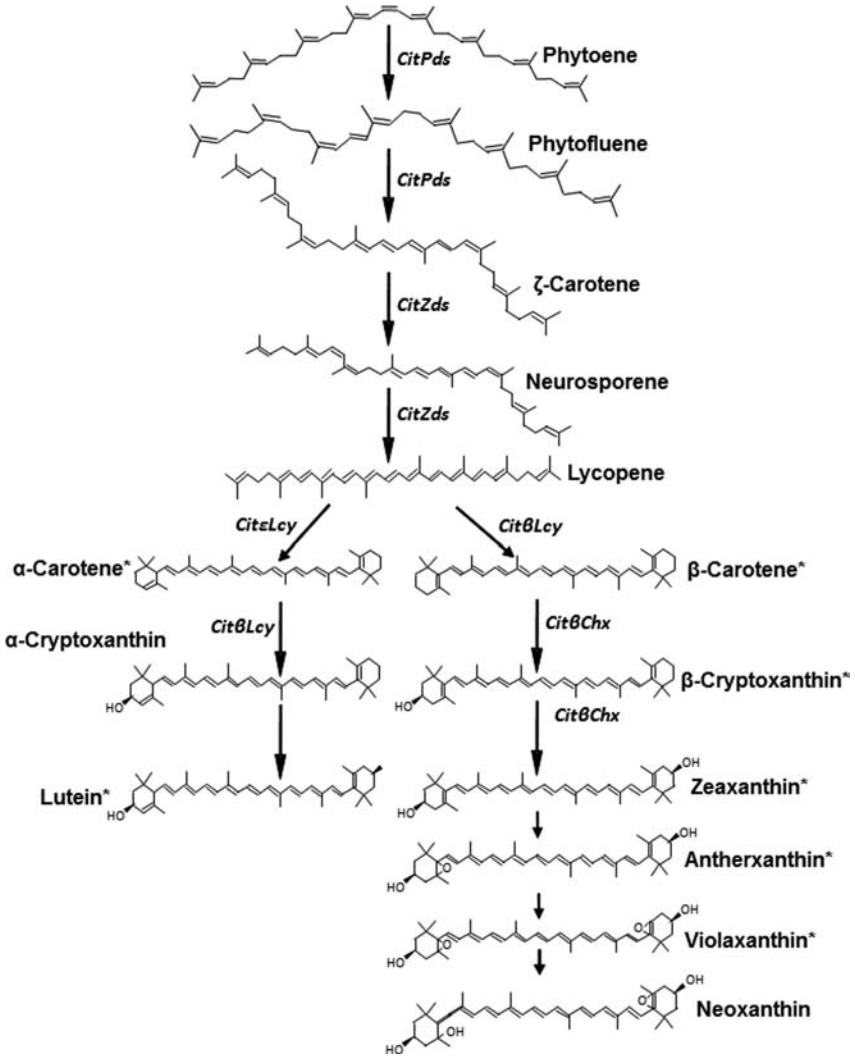

Figure 1. Schematic diagram of the carotenoid biosynthesis pathway in citrus fruit. Five partial cDNA sequences encoding citrus *Pds*, *Zds*, *ε-Lcy*, *β-Lcy*, and *β-Chx* were used to design specific primers for gene expression analyses. *, carotenoids separated by HPLC and quantified. GGPP, Geranylgeranyl pyrophosphate.

through hydroxylation of *β*-carotene to form *β*-cryptoxanthin and zeaxanthin.[9] Little information is available on carotenoid changes in late-harvested senescent citrus fruit peel.

Postharvest ethylene treatment caused chlorophyll degradation and induced changes in citrus fruit carotenoid composition and carotenoid biosynthetic pathway gene.[9,12,13] Ethylene treatment accelerated changes in carotenoid content and gene expression that occur naturally in peel during fruit maturation. In contrast to ethylene, little is known about carotenoid profile changes in peel as a result of ethephon or CMNP treatment. Ethephon is considered a practical field treatment with the gaseous phytohormone ethylene. Uptake of ethephon into plant tissue results in chemical cleavage of the molecule to yield ethylene, phosphate, and hydrochloric acid.[14] Ethylene derived from ethephon cleavage is thought to promote abscission, but phosphate has also been shown to have some effect.[15] The fact that phosphate may contribute to the response warrants investigation of carotenoid-related changes induced by ethephon.

CMNP is a compound with uncoupling activity that accelerates abscission.[16] Fruit treated with CMNP are stimulated to produce ethylene as abscission commences.[17] Although total carotenoid content increases with CMNP treatment,[6] nothing is known about specific carotenoid-related changes brought about by this compound. The purpose of this work was to compare the effect of canopy CMNP and ethephon applications on individual carotenoid composition and carotenoid biosynthesis pathway gene expression in flavedo (the colored portion of the fruit peel) in the main processing sweet orange cultivar grown in Florida, 'Valencia' (*Citrus sinensis* L. Osbeck), and to compare these changes at different maturation stages.

MATERIALS AND METHODS

Reagents and Standards. All reagents used were HPLC grade from Fisher Scientific or from Merck. One group of carotenoid standards were purchased from Sigma Chemical Co. (St. Louis, MO) and consisted of canthaxanthin, ethyl-*β*-apo-8′-carotenoate, *β*-apo-8′-carotenal, lycopene, and *β*-carotene. Another group of carotenoid standards consisting of *β*-cryptoxanthin, *α*-carotene, *β*-carotene, lutein, and zeaxanthin was obtained from Dr. Gerry Spinwall of Hoffmann-La Roche.

Plant Material, Treatments, Fruit Quality and Total Carotenoid Determination. Fruit of 'Valencia' (*C. sinensis* L. Osbeck) trees grown on rough lemon rootstock were harvested from 15-year-old trees located at the Citrus Research and Education

Center in Lake Alfred, FL. Treatments were applied during a normal harvest season at three different months in two consecutive seasons, 2005 and 2006, each representing different fruit maturation stages: early (January), middle (March), and late maturation (May) stages. Three canopy sectors of approximately 3 m^3 each were randomly selected for each treatment from a group of 10 trees. Treatments were applied with a pressurized hand sprayer to runoff. Treatments were 200 mg/L (ppm; 1.2 mM) CMNP, 400 ppm ethephon (2-chloroethylphosphonic acid; 2.8 mM), or water. Tween 20 (0.15%) was used as adjuvant in all treatments. From each replicate canopy sector, 30 fruit were selected for analysis 96 h after application. At harvest, peel color (Hunter Lab color space values *a* and *b*, and *a*/*b*) was measured with a Minolta CR-330 colorimeter on three equidistant points around the equatorial plane of each fruit. Measurements (270 individual measurements) were averaged to obtain a single value. Flavedo was removed from the equatorial one-third of each of the 30 fruit in the replicate, pooled, frozen in liquid nitrogen, and stored at −80 °C until needed for carotenoid extraction or RNA isolation. Juice was extracted from juice sacs using an electric hand reamer to avoid cross-contamination from flavedo exudates and filtered through a metal sieve with a pore size of 0.6 mm, and juice analysis was performed as previously described.[18] Total carotenoids were extracted from 1g of flavedo and analyzed spectrophotometrically at 450 nm according to Eilati et al.,[19] with some modification.[6] Total carotenoid content in flavedo and juice was calculated according to the formulas mg/g = ($DO^{450\ nm}$ × mL of *n*-hexane × 1.11 × 100 × dilution)/(2500 g sample) and μg/mL = ($DO^{450\ nm}$ × mL of *n*-hexane × 100000 × dilution)/(2500/mL sample), respectively, and expressed as *β*-carotene.

Total RNA Extraction and Real-Time Polymerase Chain Reaction Analysis of Selected Carotenoid Biosynthesis Genes. Total RNA from flavedo was isolated using trizole, and contaminating DNA was digested with RNase-free DNase (Qiagen, Valencia, CA). Two-step real-time reverse transcription polymerase chain reaction (RT-PCR) was performed. First-strand cDNA was synthesized from 1 μg of total RNA by random priming with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed in an ABI PRISM 7500 Sequence Detection Fast System (Applied Biosystems, Foster City, CA). Analysis was performed on 1 μL of diluted cDNA in a final volume of 20 μL using the SYBR Green PCR kit (Applied Biosystems, Foster City, CA). PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analyses were performed to confirm target-specific amplification. Ten- to 10000-fold dilutions of cDNA were made in 20-μL reactions and used for all primer sets to calculate the real-time efficiency of each sample during RT-PCR. Amplification reactions were performed with all primer sets during the same run. *Citrus sinensis* GAPDH was used as internal control. A citrus actin sequence was also used for confirmation with similar results. The relative expression ratios of target genes (*Pds*, *Zds*, *ε-Lcy*, *β-Lcy*, and *β-Chx*) were calculated in comparison to that of the internal control by the $\Delta\Delta C_T$ method using software package SDS 1.3.1 from the ABI PRISM 7500 Sequence Detection Fast System. Three biological replicates, each containing tissue pooled from the three fruit replicates were utilized and all analyses were performed in duplicate. Repetitive measurements were averaged to obtain a single value for each replication. The following specific primers were designed against available *C. sinensis* sequences in Genbank by using Genexpress software package and used for analysis: *GAPDH* (forward 5′-GGAAGGTCAAGATCGCAATCAA-3′ and reverse 5′-CGTCCCTCTGCAAGATGACTCT-3′), *Pds* (forward 5′-AGCCGATTTGATTTTCCTGAAG-3′ and reverse 5′-GCCAAGTCAGCATTTCATTATTC-3′), *Zds* (forward 5′-GGAAGGAGCAACTTTGTCTGGTA-3′ and reverse 5′-GCTGCTTCCTCAGTGCTACTAATTC-3′), *ε-Lcy* (forward 5′-GGACTAGCACTCATTTTGCAACTG-3′ and reverse 5′-CAAGGAAACCGTGCCACATC-3′), *β-Lcy* (forward 5′-CAGCCGGCCTCCGTCTA-3′ and reverse 5′-GGTGAAGGATCA-ACACAACATACC-3′), and *β-Chx* (forward 5′-CCACGATGGTCT-CGTTCACA-3′ and reverse 5′-ATAAGGCACGTCGGCAATG-3′). Accession numbers of sequences are CX668060 (*GAPDH*), AB114649 (*Pds*), AB114650 (*Zds*), AB114655 (*ε-Lcy*), AB114652 (*β-Lcy*), and AB114653 (*β-Chx*).

HPLC Analysis of Flavedo and Juice Carotenoids. Flavedo (150 mg) was ground in liquid nitrogen to a fine powder using a mortar and pestle. Juice was extracted with an electric reamer. Powder and juice were extracted in 10 mL of cold 100% acetone and vigorously shaken to solubilize carotenoids. The supernatant was decanted, transferred to a separatory funnel, and washed with petroleum ether and water (1:1, v/v). The petroleum ether layer was collected and evaporated to dryness under vacuum at room temperature. The solid residue was dissolved in 6 mL of diethyl ether and 6 mL of 10% methanolic KOH and kept overnight at room temperature in the absence of light to achieve full saponification of extracted carotenoids. The slurry was extracted with 20 mL of diethyl ether and 100 mL of 10% NaCl solution. After discarding the chlorophyll-containing aqueous layer, the ether fraction was washed with distilled water to remove alkali, dried over anhydrous sodium sulfate, and evaporated to dryness under vacuum. Carotenoids were dissolved in 0.5 mL of methyl *tert*-butyl ether (MTBE), diluted in 1 mL of absolute methanol, and stored in sealed amber vials at −20 °C until analysis. Chromatography was performed with a Hewlett-Packard 1090 M microprocessor-controlled integrated chromatographic system as previously described. The column used was a 4.6 × 250 mm, 5 μm C30 carotenoid column (YMC/Waters, Milford, MA).[20] Solvent composition for carotenoid elution and analysis consisted of MeOH, water, and MTBE with a ternary gradient elution program as previously reported. The photodiode array detector was set to scan from 250 to 540 nm throughout the elution profile. Data were acquired and analyzed by using the Xcalibur software package (Thermo Electron Corp., Waltham, MA). Chromatographic peaks were identified by published specific retention times, order of elution, and absorption spectra.[20] The carotenoid peaks were integrated at their individual maxima wavelength and their content was calculated by reference to calibration curves prepared for each carotenoid. For the purposes of this study, carotenoids that had their central absorbance peak at 440 nm or greater were considered red carotenoids and consisted of antherxanthin, *cis*-antherxanthin, *β*-carotene, *β*-cryptoxanthin, and zeaxanthin. Total yellow carotenoids were computed by summing chromatographic peak areas for *α*-carotene, lutein, and *cis*-violaxanthin.

Statistical Analysis. Flavedo color, juice quality (acidity, soluble solids, and degrees Brix), and total carotenoid content in flavedo during maturation were subjected to analysis of variance and Duncan's multiple range test using the SAS statistical package (SAS Institute Inc., Cary, NC). Individual and grouped carotenoid contents and relative gene expression were presented as means ± SE. Regression analysis for total carotenoids was run using Sigmaplot, version 10.0 (Systat Software, San Jose, CA). Slopes for regression lines were compared by using *t* test analysis with two degrees of freedom.

RESULTS

Juice Quality, Peel Color, and Total Flavedo Carotenoid Content. Juice quality followed normal trends for fruit once minimum maturity was reached; namely, sugars (°Bx) decreased slowly and acids declined more rapidly as fruit became increasingly mature (data not shown). At each harvest date, ethephon or CMNP application did not significantly change acid and Brix levels, but peel color was impacted. As maturation progressed, *a* values increased in control, ethephon, and CMNP-treated peel from negative to positive values, indicating color change from green to red, and *b* values became increasingly more positive, indicating that the peel became increasingly more yellow (Figure 2). In ethephon-treated flavedo, *b* values became increasingly positive but declined at the last maturation stage. Similar trends were measured in CMNP-treated peel. The *a*/*b* ratio went from negative to positive, indicating a change in peel color from green to orange.

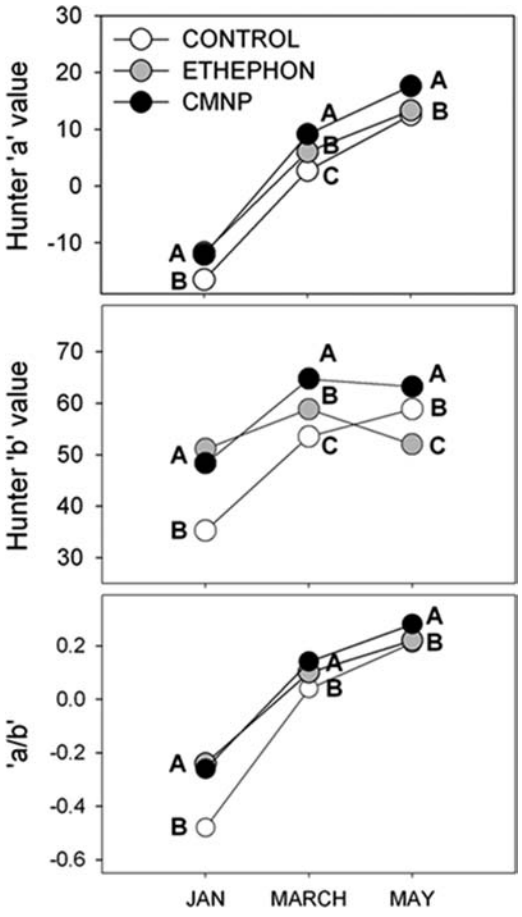

Figure 2. Hunter Lab color scale values of 'Valencia' sweet orange peel at three maturation stages: (A) *a*, (B) *b*, and (C) *a*/*b*. Within each harvest date and color scale value, means followed by the same letter are not significantly different, $p \leq 0.05$.

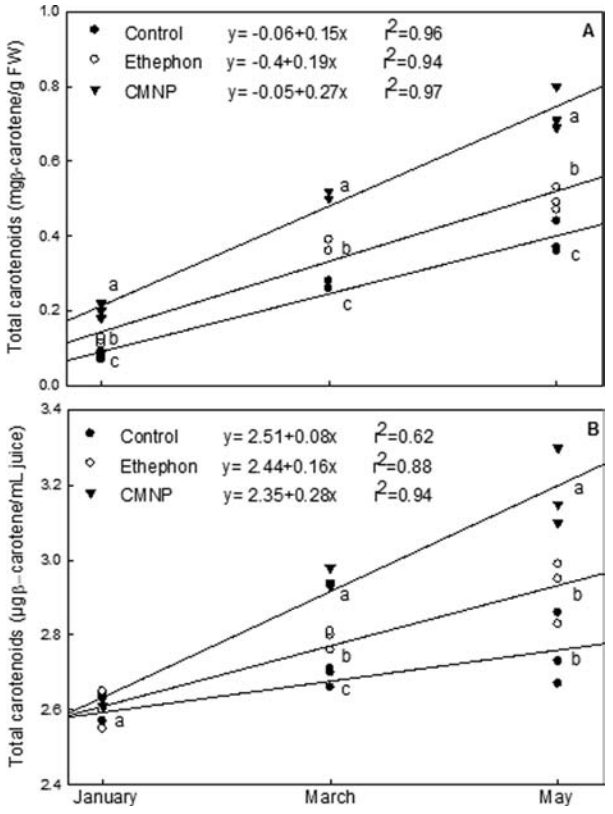

Figure 3. Total carotenoid content in 'Valencia' sweet orange flavedo (A) and juice (B) from fruit treated with water (control), 400 ppm ethephon, or 200 ppm CMNP at three harvest dates representing three maturation stages. Regression equations and coefficients are shown for each treatment. Within each harvest date, means followed by the same letter are not significantly different, $p \leq 0.05$.

The rate of natural and abscission-agent-induced color change was greatest in fruit at early to midmaturation stages. Fruit treated with CMNP produced significantly higher *a*, *b*, and *a*/*b* values at all maturation stages when compared with control. Ethephon-treated fruit at middle to late maturation stages had significantly lower *a* and *b* peel values when compared with CMNP-treated fruit ($p \leq 0.05$). Total carotenoid content was significantly greater in CMNP-treated flavedo when compared with ethephon-treated flavedo (Figure 3A) and juice (Figure 3B), and both were significantly greater than the control at all maturation stages. The rate of maturation-dependent carotenoid accumulation was significantly greater ($p \leq 0.05$) in flavedo of fruit treated with CMNP than ethephon or control flavedo. The pattern of total carotenoid accumulation and the effect of abscission application was similar in juice.

Expression of Carotenoid Biosynthetic Genes. To examine how abscission agent application may regulate changes in carotenoid content, mRNA accumulation of five carotenoid biosynthetic genes in flavedo during maturation was measured. We selected key genes important in the regulation of carotenoid accumulation, including the early steps of the pathway (*Pds* and *Zds*) shown to be regulated during maturation,[10] the cyclization branch point (*β-Lcy* and *ε-Lcy*) important for directing carotenoid flux to the *β*,*ε*- and *β*,*β*-branch, and *β*-carotene hydroxylation (*β-Chx*) shown to direct carotenoid flux toward violaxanthin formation at latter stages of maturation. Small increases in *Pds* and *Zds* gene expression were measured in control flavedo as maturation progressed (Figure 4). Ethephon application increased expression of these genes from early to midmaturation stages; however, no change or a decline in *Pds* and *Zds* was measured from middle to late maturation stage, respectively. CMNP activated *Pds* and *Zds* gene expression from early to midmaturation stages. As maturation progressed to the late stage, increased *Pds* expression was measured but *Zds* declined. *ε-Lcy* and *β-Lcy* expression declined in controls as maturation proceeded. Small maturation-dependent increases in *ε-Lcy* expression were measured in fruit flavedo treated with ethephon, whereas ethephon application increased and then decreased *β-Lcy* expression. CMNP application markedly increased *ε-Lcy* and *β-Lcy* expression in flavedo of fruit from the midmaturation stage (15- and 45-fold, respectively) but declined to near-early maturation levels in late maturation stage flavedo. CMNP induced small changes in *β-Chx* expression as maturation progressed, but ethephon application markedly increased expression in flavedo from middle and late maturation stages (14- and 12-fold, respectively). No significant change in expression occurred in control peel. Patterns of *ε-Lcy*, *β-Lcy*, and *β-Chx* expression induced by CMNP and ethephon suggested that yellow and red carotenoid composition might be differentially altered.

Carotenoid Profile in Flavedo. In 'Valencia' flavedo, eight carotenoids were identified (Table 1). Antheraxanthin, *cis*-antheraxanthin, *β*-carotene, *β*-cryptoxanthin, and zeaxanthin

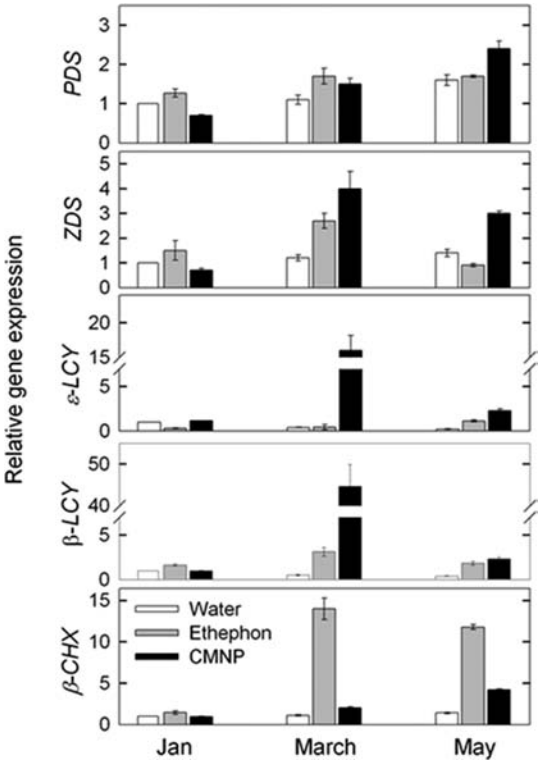

Figure 4. Relative expression of carotenoid biosynthetic genes *Pds*, *Zds*, *ε-Lcy*, *β-Lcy*, and *β-Chx* in 'Valencia' sweet orange flavedo treated with water (control), 400 ppm ethephon, and 200 ppm CMNP at three harvest dates representing three maturation stages. Bars represent means, and lines through bars show the SE_{mean} of three biological replicates.

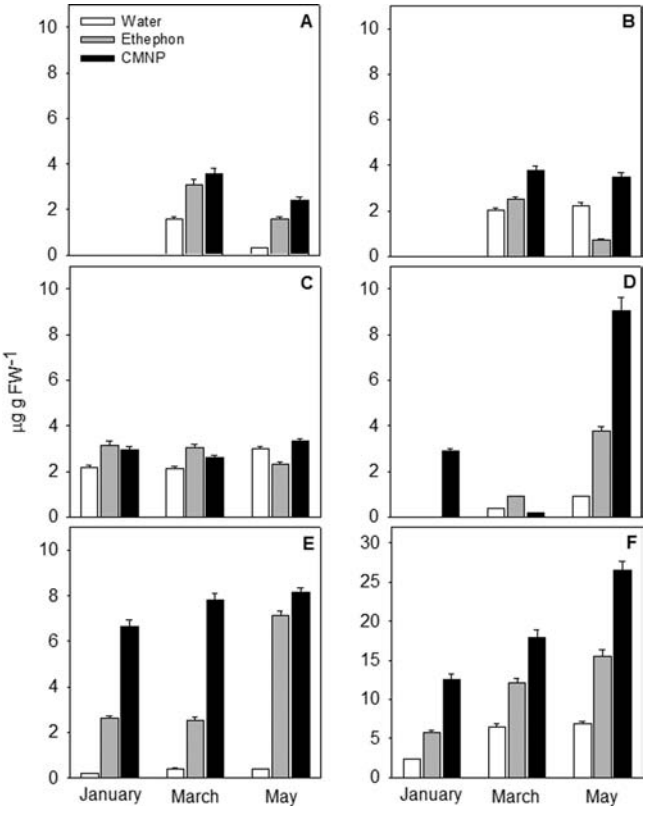

Figure 5. Accumulation of *β*-carotene (A), *β*-cryptoxanthin (B), zeaxanthin (C), antherxanthin (D), *cis*-antherxanthin (E), and total red carotenoids (F) in 'Valencia' sweet orange flavedo treated with water (control), 400 ppm of ethephon, and 200 ppm of CMNP at three harvest dates representing three maturation stages. Data are means and SE_{mean} of three biological replicates.

were selected as red carotenoids and *α*-carotene, lutein, and violaxanthin as yellow carotenoids based on published and measured absorption spectra.[20] In general, zeaxanthin, antherxanthin, and *cis*-antherxanthin were the most abundant red carotenoids (Figure 5), whereas violaxanthin was the most abundant yellow carotenoid (Figure 6). Individual red carotenoids in flavedo fluctuated during maturation, but the abundance of most of these carotenoids was greater in the late stage when compared to the early stage (Figure 5). CMNP treatment resulted in higher accumulation of each red carotenoid when compared with controls. Red carotenoids induced by ethephon application were less abundant but followed the same pattern as CMNP, with notable exceptions. Zeaxanthin and *cis*-antherxanthin increased in flavedo from late stage fruit harvested. When considered together, CMNP accumulated more total red carotenoids than ethephon or controls as maturation progressed. As maturation advanced, yellow carotenoids *α*-carotene and *cis*-violaxanthin decreased, whereas lutein remained steady; however, a slight increase in *α*-carotene was measured in CMNP-treated flavedo from the midmaturation stage as compared to water and ethephon-treated fruit (Figure 6). In general, flavedo of fruit treated with abscission agents had less or similar amounts of these yellow carotenoids when compared with controls. Total yellow carotenoids decreased as maturation progressed.

Table 1. Spectroscopic Characteristics of Relevant Carotenoids Identified in 'Valencia' Sweet Orange Flavedo after CMNP Treatment[a]

		observed (nm)			literature (nm)		
t_R[b] (min)	carotenoid	peak I	peak II	peak III	peak I	peak II	peak III
10.15	antherxanthin	420	443	472	418	442	470[30]
11.13	*cis*-antherxanthin	419	442	472	416	441	468[31]
16.02	*cis*-violaxanthin	418	439	469	414	437	464[31]
22.93	lutein	425	448	475	423.5	445.5	473.5[32]
26.92	zeaxanthin	428	453	480	425.5	451.5	477.5[32]
36.23	*β*-cryptoxanthin	423	448	474	426	452	478[31]
39.12	*α*-carotene	423	444	471	423.5	445.5	473.5[32]
41.35	*β*-carotene	425	449	477	429.5	451.5	477.5[30]

[a]Data are from the May harvest date representing the late maturation stage. [b]retention time.

dx.doi.org/10.1021/jf305359x | J. Agric. Food Chem. 2013, 61, 3082−3089

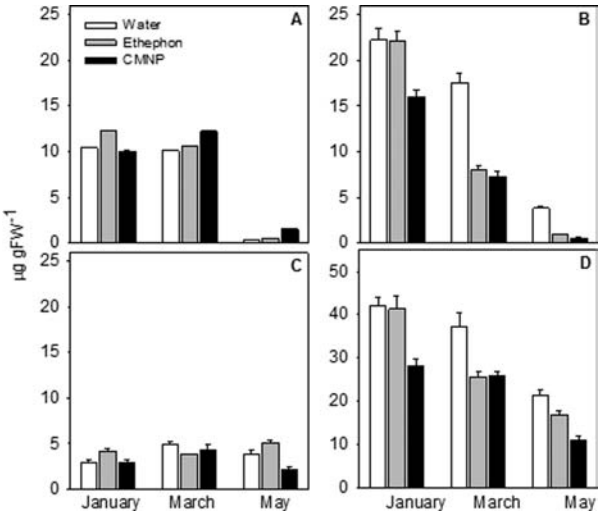

Figure 6. Accumulation of α-carotene (A), *cis*-violoxanthin (B), lutein (C), and total yellow carotenoids (D) in 'Valencia' sweet orange flavedo treated with water (control), 400 ppm of ethephon, and 200 ppm of CMNP at three harvest dates representing three maturation stages. Data are means and SE_{mean} of three biological replicates.

DISCUSSION

Application of abscission agents to mature citrus fruit cause several changes that alter fruit quality. Internal juice quality was not affected, since abscission agent application did not alter the pattern or intensity of juice quality changes in percent acid and degree Brix typical during natural maturation. [21] Visual difference in peel color was the most striking change in external fruit quality caused by abscission agent treatment. Fruit treated with CMNP appeared more yellow and/or orange than ethephon or control fruit at any harvest date, and this was reflected in higher Hunter Lab color values *a*, *b*, and *a*/*b*. Acceleration of color change suggests that CMNP and ethephon advanced senescence in mature fruit peel.[6,22]

Trends in carotenoid changes during growth and development of flavedo reported in our work confirm changes reported by others.[23,10,11] CMNP was more effective in increasing total flavedo carotenoid content at all harvest dates, and the rate of accumulation throughout maturation was higher. Previous work demonstrated that CMNP was more effective than ethephon at increasing accumulation of total carotenoids in 'Hamlin' flavedo, an early maturing citrus variety.[6] Moreover, similar total carotenoid accumulation patterns were measured in juice extracted from CMNP- and ethephon-treated fruit (Figure 3B). These data suggest that both compounds cause maturation-dependent carotenoid changes in juice sacs as a result of components or signals generated in peel tissue.

The natural phytohormone ethylene may play a role in abscission-agent-induced color change. Ethephon directly releases ethylene upon uptake in treated tissues,[15] whereas CMNP causes treated tissues to produce ethylene.[18] Ethylene and other growth regulators accelerate color change in citrus fruit by promoting chlorophyll degradation, resulting in unmasking carotenoids and other chromophores contained within the plastid, and induce carotenoid biosynthesis.[24,12,13,9] On the other hand, CMNP has uncoupling activity and is thought to induce various stresses, including wounding.[6,16] Environmental stress has been shown to induce carotenoid biosynthetic gene expression and differential accumulation of carotenoids in pepper and tomato.[25,26] CMNP may induce greater total carotenoid accumulation in flavedo and juice through ethylene- and stress-induced carotenoid biosynthesis. The decrease in violaxanthin content observed in latter stages of maturation was enhanced by ethephon, but a more pronounced decrease was observed after CMNP treatment, consistent with data reported in 'Satsuma' mandarins treated with ethephon and in 'Navelate' oranges treated with ethylene.[9,27] This data suggests that metabolism of violaxanthin to ABA may be enhanced by CMNP as a stress response similarly to that observed in 'Navelate' oranges upon NCED gene activation in response to dehydration and supports previous results showing increase of ABA content in flavedo after CMNP treatment.[28,29] Changes in carotenoid biosynthesis pathway gene expression suggested that both abscission agents differentially regulated key pathway components. Ethephon and CMNP affected *Pds* and *Zds* transcript expression in a similar manner, suggesting the potential for downstream carotenoid changes. When applied to fruit at the midmaturation stage, CMNP induced marked accumulation of β- and ε-*Lcy* transcripts, whose protein products have key roles in formation of downstream red and yellow carotenoids. Ethephon minimally changed β- and ε-*Lcy* expression when applied at any harvest date. However, ethephon markedly accelerated β-*Chx* expression in middle and late maturation stages, whereas CMNP had little effect. These results indicate the potential for CMNP and ethephon to cause differential accumulation of carotenoids at the middle and late maturation stages. However, carotenoid accumulation was not necessarily associated with gene expression or measurable color change. This lack of correlation between gene expression and carotenoid accumulation following different treatments has been shown previously by other researchers. Lack of correlation observed in gene expression profile and carotenoid accumulation in both flavedo and juice sacs have been attributed to post-transcriptional factors such as enzymatic properties that may be affected by stress factors like temperature;[27] also, other genes from upstream metabolic pathways may be involved in the regulation of carotenoid accumulation in citrus fruit.[33] Accumulation of more total flavedo carotenoids by CMNP application at all maturation stages was supported by induction of the β,ε- and β,β-branches of the pathway, and total red carotenoids derived by β,β-branch carotenoid metabolism were increased. The increasingly positive Hunter Lab color value *a*, indicating peel color change from green to red, was consistently greater in CMNP-treated fruit at all maturation stages and correlated well with trends in total red carotenoids. Total yellow carotenoids derived by metabolism through the β,ε-branch of the pathway were less in CMNP-treated flavedo and declined as maturation progressed, even though the Hunter Lab color value *b* increased with increasing maturation and was generally greater than ethephon or control treatments.

Upregulation of β-*Chx* has been observed in harvested fruit treated with ethylene during postharvest storage and during natural maturation of 'Navelate' and 'Valencia' oranges.[9−11] In our work, ethephon preferentially induced β-*Chx* expression, suggesting the potential for altered metabolite movement through the β,β-branch of the pathway, yet total red carotenoids were less abundant than those in CMNP-treated flavedo. This may be explained by assuming downstream metabolism of the β-*Chx* protein product to nonred carotenoids or unrelated compounds. The decline in zeaxanthin and β-cryptoxanthin in ethephon-treated flavedo shown in this

work would be expected if increased metabolism of β,β-branch carotenoids occurred.

In conclusion, abscission agent application accelerated peel color change, altered carotenoid content, and differentially changed *β-Lcy*, *ε-Lcy*, and *β-Chx* gene expression as fruit maturation progressed. More intensely colored fruit peel may not be an important commercial advantage of abscission application in fruit destined for the processed market; however, similar trends measured in juice total carotenoids may serve to enhance color and nutritional benefit. Nevertheless, changes in carotenoid accumulation and expression patterns of carotenoid biosynthesis genes in flavedo suggest that regulation of carotenoid accumulation may be under transcriptional, translational, and post-translational control.

AUTHOR INFORMATION

Corresponding Author

*E-mail: alferez@iata.csic.es.

Funding

F.A was supported by Ramon y Cajal program (MICINN and Fondo Social Europeo).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of Mr. Jack Smoot.

REFERENCES

(1) Burns, J. K.; Buker, R. S.; Roka, F. M. Mechanical harvesting capacity in sweet orange is increased with an abscission agent. *HortTechnology* **2005**, *15*, 758−765.

(2) Hartmond, U.; Yuan, R.; Burns, J. K.; Grant, A.; Kender, W. J. Citrus fruit abscission induced by methyl jasmonate. *J. Am. Soc. Hortic. Sci.* **2000**, *125*, 547−552.

(3) Burns, J. K.; Pozo, L. V.; Arias, C. R.; Hockema, B.; Rangaswamy, V.; Bender, C. L. Coronatine and abscission in citrus. *J. Am. Soc. Hortic. Sci.* **2003**, *128*, 309−315.

(4) Kender, W. J.; Hartmond, U.; Burns, J. K. Fruit abscission and leaf drop in citrus cultivars treated with metsulfuron-methyl. *HortTechnology* **1999**, *9*, 412−416.

(5) Burns, J. K. Using molecular biology tools to identify abscission materials for citrus. *HortScience* **2002**, *37*, 459−464.

(6) Alferez, F.; Pozo, L.; Burns, J. K. Physiological changes associated with senescence and abscission in mature citrus fruit induced by 5-chloro-3-methyl-4-nitro-1*H*-pyrazole and ethephon application. *Physiol. Plant.* **2006**, *127*, 66−73.

(7) Oberholster, R.; Cowan, K.; Molnar, P.; Toth, G. Biochemical basis of color as an aesthetic quality in *Citrus sinensis*. *J. Agric. Food Chem.* **2001**, *49*, 303−307.

(8) Gross, J. *Carotenoids: Pigments in Fruits*. Academic Press: London, 1987; p 303.

(9) Rodrigo, M. J.; Zacarias, L. Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic genes in the flavedo of orange (*Citrus sinensis* L. Osbeck) fruit. *Postharvest Biol. Technol.* **2007**, *43*, 14−22.

(10) Kato, M.; Ikoma, Y.; Matsumoto, H.; Sugiura, M.; Hyodo, H.; Yano, M. Accumulation of carotenoids and expresión of carotenoid biosynthetic genes during maturation in citrus fruit. *Plant Physiol.* **2004**, *134*, 824−837.

(11) Rodrigo, M.; Marcos, J. F.; Zacarias, L. Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. *J. Agric. Food Chem.* **2004**, *52*, 6724−6731.

(12) Purvis, A. C.; Barmore, C. R. Involvement of ethylene in chlorophyll degradation in peel of citrus fruits. *Plant Physiol.* **1981**, *68*, 854−856.

(13) Stewart, I.; Wheaton, T. A. Carotenoids in citrus: Their accumulation induced by ethylene. *J. Agric. Food Chem.* **1972**, *20*, 448−449.

(14) Warner, H. L.; Leopold, A. C. Ethylene evolution from 2-chloroethylephosphonic acid. *Plant Physiol.* **1969**, *44*, 156−158.

(15) Goren, R.; Huberman, M.; Martin, G. C. Phosphorus-induced leaf abscission in detached shoots of olive and citrus. *J. Am. Soc. Hortic. Sci.* **1998**, *123*, 486−737.

(16) Alferez, F.; Singh, S.; Umbach, A. L.; Hockema, B.; Burns, J. K. Citrus abscission and Arabidopsis plant decline in response to 5-chloro-3-methyl-4-nitro-1*H*-pyrazole are mediated by lipid signaling. *Plant Cell Environ.* **2005**, *28*, 1436−1449.

(17) Yuan, R.; Burns, J. K. Temperature factor affecting the abscission response of mature fruit and leaves to CMN-pyrazole and ethephon in 'Hamlin' oranges. *J. Am. Soc. Hortic. Sci.* **2004**, *129*, 287−293.

(18) Pozo, L.; Yuan, R.; Kostenyuk, I.; Alferez, F.; Zhong, G. Y.; Burns, J. K. Differential effects of 1-methylcyclopropene on citrus leaf and mature fruit abscission. *J. Am. Soc. Hortic. Sci.* **2004**, *129*, 473−478.

(19) Eilati, S. K.; Monselise, S. P.; Budowski, P. Seasonal development of external color and carotenoid content in the peel of ripening Shamouti oranges. *J. Am. Soc. Hortic. Sci.* **1969**, *94*, 346−348.

(20) Eilati, S. K.; Budowski, P.; Monselise, S. P. Xanthophyll esterification in the flavedo of citrus fruit. *Plant Cell Physiol.* **1972**, *13*, 741−746.

(21) Rouseff, R.; Raley, L.; Hofsommer, H. Application of a diode array detection with a C-30 reversed phase column for the separation and identification of saponified orange juice carotenoids. *J. Agric. Food Chem.* **1996**, *44*, 2176−2181.

(22) Wardowski, W. F., Miller, W. M., Hall, D. J., Grierson, W. *Fresh Citrus Fruits*, 2nd ed; Florida Science Source, Inc.: Longboat Key, FL, 2006; p 602.

(23) Bonora, A.; Pancaldi, S.; Gualandri, R.; Fasulo, M. P. Carotenoid and ultrastructural variations in plastids of *Arum italicum* Miller fruit during maturation and ripening. *J. Exp. Bot.* **2000**, *346*, 873−884.

(24) Jacob-Wilk, D.; Holland, D.; Goldschmidt, E. E.; Riov, J.; Eyal, Y. Chlorophyll breakdown by chlorophyllase: Isolation and functional expression of *Chlase I* gene from ethylene-treated *Citrus* fruit and its regulation during development. *Plant J.* **1999**, *20*, 653−661.

(25) Bouvier, F.; Backhaus, R. A.; Camara, B. Induction and control of chromoplast-specific carotenoid genes by oxidative stress. *J. Biol. Chem.* **1998**, *273*, 30651−30659.

(26) Herde, O.; Pena-Cortes, H.; Fuss, H.; Willmitzer, L.; Fisahn, J. Effects of mechanical wounding, current application and heat treatment on chlorophyll fluorescence and pigment composition in tomato plants. *Physiol. Plant.* **1999**, *105*, 179−184.

(27) Matsumoto, H.; Ikoma, Y.; Kato, M.; Nakajima, N.; Hasegawa, Y. Effects of postharvest temperature and ethylene on carotenoid accumulation in the flavedo and juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. *J. Agric. Food Chem.* **2009**, *57*, 4724−4732.

(28) Rodrigo, M. J.; Alquezar, B.; Zacarias, L. Cloning and characterization of two 9-*cis*-epoxycarotenoid dioxygenase genes, differentialy regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L.Osbeck). *J. Exp. Bot.* **2006**, *57*, 633−643.

(29) Yuan, R.; Hartmond, U.; Grant, A.; Kender, W. J. Physiological factors affecting response of mature 'Valencia' orange fruit to CMN-pyrazole. II. Endogenous concentrations of indole-3-acetic acid, abscisic acid and ethylene. *J. Am. Soc. Hortic. Sci.* **2001**, *126*, 420−426.

(30) Farin, D.; Ikan, R.; Gross, J. The carotenoid pigments in the juice and flavedo of a mandarin hybrid (*Citrus reticulata* cv. Michal) during ripening. *Phytochemistry* **1983**, *22*, 403−408.

(31) De Ritter, E.; Purcell, A. E. Carotenoid analytical methods. In *Carotenoids as Colorants and Vitamin A Precursors*; Bauernfeind, J. C., Ed.; Academic Press: London, 1981, pp 815−923.

(32) Van Heukelem, L.; Lewitus, A.; Kana, T. M. High-performance liquid chromatography of phytoplankton pigments C18 column. *J. Phycol.* **1992**, *28*, 867−872.
(33) Alós, E.; Cercós, M.; Rodrigo, M. J.; Zacarías, L.; Talón, M. Regulation of color break in citrus fruits. Changes in pigment profiling and gene expression induced by gibberellins and nitrate, two ripening retardants. *J. Agric. Food Chem.* **2006**, *54*, 4888−4895.