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ARTICLE

Physiological and Molecular Analysis of the Maturation Process in Fruits of Clementine Mandarin and One of Its Late-Ripening Mutants

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Peel color is one of the main features affecting citrus quality. Clementine is a widespread citrus species with several mutants showing a delay in pigmentation and harvesting. This work characterizes the fruit development and ripening of two clementine clones, 'Comune', a widespread variety, and one of its natural mutations, 'Tardivo', which differ by a delayed color-break and extended harvest period. Morphological, chemical, and molecular analyses were carried out on fruits of both genotypes during the whole maturation process. Analysis showed that mutation did not affect ripening characteristics such as juice acidity and TSS. However, biochemical and molecular analysis revealed marked differences in the flavedo regarding carotenogenesis and chlorophyllase gene expression. Carotenoid showed quantitative differences at biochemical and molecular levels. Results demonstrated that the mutation in 'Tardivo' influenced the transcriptional activation of *PSY*, a key step in carotenoid biosynthesis. The differential *PSY* expression led to a significant quantitative difference in phytoene accumulation between the two genotypes. Also, 'Tardivo' showed delayed accumulation of carotenes, lutein, and β,β-xanthophylls. The differential expression of genes involved in ethylene biosynthesis and perception suggested differing responses to ethylene signaling between the two genotypes. Moreover, exogenous application of ethylene revealed a different sensitivity of the two varieties to this hormone. The analysis added new information to better understand the complex process of ripening in citrus.

KEYWORDS: Citrus; chlorophyllase; carotenogenesis; HPLC; ethylene

INTRODUCTION

Fruit development and ripening are complex processes involving physiological and biochemical changes that are under hormonal, nutritional, and environmental control (*1*). In citrus, one of the most evident phenomena in late maturation is the change in peel (flavedo) color resulting from massive accumulation of carotenoids together with chlorophyll degradation (*2*). Carotenoid concentration and composition vary greatly among citrus varieties and depend on growing conditions.

The relationship between carotenoid biosynthetic gene expression and carotenoid accumulation during fruit maturation has been previously investigated in citrus. Kato et al. (*3*), analyzing Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*Citrus sinensis* L. Osbeck), and Lisbon lemon (*Citrus limon* Burm.f.), suggested that carotenoid accumulation was highly regulated by coordination between different carotenoid biosynthetic genes. These results have been generally confirmed in other studies (*4*−*9*).

Citrus color-break is regulated by metabolic signals, including ripening inducers such as ethylene and sucrose and ripening retardants such as gibberellins and nitrogen (*4*, *10*). Most knowledge about the regulation of external color change in citrus has resulted from the exogenous application of various chemicals, such as hormones (ethylene first) and nutrients.

Ripening-related genes involved in chlorophyll degradation (*11*), carotenoid biosynthesis (*4*, *5*, *12*, *13*), and ethylene biosynthesis and perception (*14*) have been isolated and characterized in *Citrus* species. The relationship between ethylene and ripening has been intensively studied in both climacteric and nonclimateric fruits. Citrus fruits are nonclimateric, usually producing low amounts of ethylene throughout their maturation and ripening (*15*, *16*). Exogenous ethylene treatments stimulate ripening by accelerating respiration and inducing changes in peel pigmentation (chlorophyll degradation as well as carotenoid biosynthesis) (*17*−*20*). Endogenous ethylene may be required for the degreening of fruit peel, which occurs during natural fruit ripening (*19*). However, the role of ethylene in regulating natural

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citrus color-break is still unclear (*14*). Recently, ethylene treatments were correlated to carotenoid accumulation establishing the up-regulation of carotenoid biosynthetic genes in *C. sinensis* L. Osbeck (*21*). The authors suggested that ethylene application accelerates the biochemical and gene expression changes which naturally occur during ripening. Thus, even if citrus fruits produce extremely low amounts of ethylene during their development, complex regulations of ethylene production and perception might exist through the ripening period.

The characterization of late-ripening mutants with delayed color change is a useful experimental system to identify and describe the molecular mechanisms involved in maturation. This aspect could be very important for citrus fruits because improving their nutritional features and extending their harvest period might improve their marketability.

In this study, we examined the carotenoid accumulation and expression of the genes responsible for chlorophyll breakdown, carotenoid biosynthesis, and ethylene synthesis and perception during the fruit development of two clementine genotypes, 'Comune' and 'Tardivo'. 'Tardivo' clementine is a natural bud mutation of 'Comune', which differs mainly in having delayed fruit pigmentation and an extended harvest period (*22*, *23*). 'Comune' ripens fully in December, whereas 'Tardivo' is about a month later. The main aim of our study was to characterize ripening in both varieties by analyzing carotenoid at the biochemical and molecular level, chlorophyllase gene expression, and ethylene biosynthesis and perception. The results obtained from biochemical and molecular analysis added new information to better understand the complex process of ripening in citrus.

MATERIALS AND METHODS

Plant Material. Two clementine (*C. clementina* Hort. Ex Tanaka) genotypes, 'Comune' and 'Tardivo', with ripening differences were used in this study. Plants of both genotypes of the same age, grafted on sour orange (*C. aurantium* L.), were cultivated in the experimental fields of the University of Catania and subjected to standard cultural practices; fruits at different development stages were harvested monthly. The sampling periods ranged from October 1 to January 1 for 'Comune' and from October 1 to March 1 for 'Tardivo' over three years.

Physical and Chemical Measurements. Physical (weight, diameter, and external color) and chemical [juice acidity and total soluble solid (TSS)] parameters were determined monthly. Three replicate samples of 10 fruits of each variety were used for analysis. Fruit color was measured through the CIE *L*a*b** system using a Minolta chroma-meter CR-200 (Minolta, Ramsey, NJ). Fruit juice was extracted with a household electric juice extractor, centrifuged, and immediately analyzed. The total acidity of the juice, determined by titration with 0.1 N NaOH, was used as an indirect measurement of the citric acid concentration and expressed as grams of citric acid per 100 mL (*24*, *25*); the soluble solid content was determined by digital refractometry, using an Atago model PR-101 and expressed as °Brix.

Quantification of Carotenoids and HPLC Analysis. Standards were obtained from Extrasynthese (Genay, France) and from CaroteNature (Lupsingen, Switzerland). HPLC grade solvents were obtained from Baker (Deventer, The Netherlands). All other reagents were of analytical grade and supplied by Carlo Erba (Milan, Italy).

Three aliquots of flavedo (ca. 5 g) obtained from 10 fruits previously ground to a fine powder under liquid nitrogen were mixed for 20 min with 50 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v). The organic phase containing carotenoids was recovered and then used for analyses after suitable dilution with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring absorbance at 450 nm in a Varian Cary 100 spectrophotometer (Mulgrave, Victoria, Australia). Total carotenoids were calculated according to the method of De Ritter and Purcell (*26*) using an extinction coefficient of β-carotene of $\varepsilon_{1\%} = 2505$. The analyses were performed in triplicate for each sample.

Carotenoid extraction was carried out according to the Matsumoto et al. (*27*) method. Three aliquots of flavedo (ca. 3 g) obtained from 10 fruits were homogenized in 50 mL of 40% (v/v) aqueous methanol containing 0.5 g of basic magnesium carbonate using a homogenizer (Waring 8010E, Torrington, CT). After vacuum filtration, the pigment in the residue was extracted with diethyl ether/MeOH (7:3, v/v) containing 0.1% (w/v) BHT until the residue was colorless. The extract was transferred to a separator funnel containing 100 mL of diethyl ether. After the ether phase was evaporated, the residue was dissolved with 10 mL of diethyl ether, mixed with 5 mL of 20% methanolic KOH, and placed for 18 h in the dark. The alkaline mixture was transferred to a separator funnel containing 100 mLof diethyl ether. The ether phase was washed with NaCl-saturated water and treated with anhydrous Na_2SO_4. After the ether phase was evaporated, the residue was topped up to 1 mL with MTBE/MeOH (1:1, v/v) containing 0.1% BHT. The sample was filtered through a 0.2 μm filter (VWR International, Milan, Italy) and stored at −30 °C before injection into HPLC. Twenty microliters of the solution was injected.

The carotenoid separation was carried out using a C30 carotenoid column (250 × 4.6 mm i.d., 5 μm) (YMC Inc., Wilmington, NC) in accordance with the procedure of Rouseff and Raley (*28*). Initial solvent composition consisted of 90% MeOH, 5% water, and 5% MTBE. Solvent composition changed in a linear fashion to 95% MeOH and 5% MTBE at 12 min; during the next 8 min (20 min running time) the solvent composition was changed to 86% MeOH and 14% MTBE. After this concentration had been achieved, the solvent was gradually changed to 75% MeOH and 25% MTBE at 30 min. The final composition was reached at 50 min and consisted of 50% MeOH and 50% MTBE. Initial conditions were re-established in 2 min and re-equlibrated for 15 min before the next injection. The flow rate was 1 mL/min. Injection volume was 20 μL. Chromatography was carried out with a Waters Alliance liquid chromatography system equipped with a quaternary pump, a photodiode array detector, an autosampler, and Empower Manager. The analyses were performed in triplicate for each sample.

The carotenoids were identified using LC-DAD by comparing the retention times and UV−vis absorption spectra and chromatography with standards at λ 450 nm for all carotenoids, except phytoene identified at λ 300 nm.

Extraction of Total RNA and cDNA Synthesis. Flavedo samples of 'Comune' and 'Tardivo' clementines were collected every month (between October and March) during fruit development and ripening. Bulks of fruit peel were immediately stored in liquid nitrogen after collection and used for total RNA extraction using an RNeasy Plant Mini Kit according to the producer's manual (Qiagen, Hilden, Germany). Three different extractions were performed. Total RNA was treated with an RNase-Free DNase set (Qiagen) according to the manufacturer's instructions. Reverse transcriptase PCR was performed using Ready-to-Go RT-PCR beads (GE Healthcare Technologies, Little Chalfont, U.K.) with primer dT.

Real-Time Quantitative RT-PCR. To identify the genes involved in chlorophyll catabolism, carotenoid biosynthesis, and ethylene biosynthesis and signal transduction, specific primers were designed according to the sequences deposited in GenBank (**Table 1**) using Primer 3 software (*29*) and purchased from MWG Biotech (Ebersberg, Germany).

Transcript levels were determined by real-time quantitative PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and the Power SYBR Green PCR Master Mix as recommended by the manufacturer. PCR reactions were carried out in triplicate on 96-well plates (25 μL per well) in a buffer containing 1× SYBR Green (including Taq polymerase, dNTPs, SYBR Green dye) and 200 nM of each primer (forward and reverse). After 10 min of denaturing at 95 °C, two-step amplification occurred: 15 s of denaturing at 95 °C and 1 min of annealing/extension at 60 °C, with a total of 40 cycles. Identical thermal cycling was used for all targets. The transcript level was calculated by standard curve and normalized against the citrus elongation factor 1-α (*EF1*) AY498567 gene as an internal control. Results from several works indicated *EF1* as one of the best usable housekeeping genes to normalize gene expression levels because it appeared uninfluenced by various phases of development and in different environmental conditions (*30*). For each gene, the first 'Comune' sample value was defined as the 1× expression level, and results were expressed as the fold increase of mRNA over this value. Negative controls without cDNA were routinely included.

Table 1. Genes with GenBank Accession Numbers and the Relative Primers Adopted in Quantitative Real-Time PCR

gene name	primers (5′−3′)	GenBank accession no.
chlorophyllase (CHLASE)	forward ACCGCTTGTGCACCTGAAG reverse TGTCCATGTGACCATAATCTGTAGC	AF160869
phytoene synthase (PSY)	forward AGGATGGACCTTAGGAAGTCAAGA reverse CTGCATTGTAGACGCTCTCTGTT	DQ109038
Z-carotene desaturase (ZDS)	forward ATGGGTTCTTCAGTTCTGTTTCC reverse TTGGGTGCATTAACACTCATATCA	AY675215
lycopene cyclase (β-LCY)	forward CCCTTCCAGTGCTTCCTCAA reverse AGTCCTTGCCACCATATAGCCAG	AY675216
violaxanthin depoxidase (VDE)	forward TTCACCCGACCAGCTATGC reverse GACATAGTCATCTGGTTCGTTCTG	AY278314
ACC synthase 1 (ACS1)	forward CCACTGTCTTCAGCTCTCCTAAG reverse TTAAACGAGTACACAATGCCAAC	AJ011095
ACC synthase 2 (ACS2)	forward CACAGTGTTCACCAAGGAGTC reverse CGAGTAAATGATACCGACCCTAA	AJ276295
ACC oxidase (ACO)	forward TGGAGCACAGAGTGGTTTCTC reverse GGATAGATCACAGCATCACTTCC	AJ297350
ethylene receptor (ETR1)	forward CAGGAGAGAAGCGGAAACAG reverse GCTCGGGTGTCAATTCAGTC	AJ276294
ethylene receptor (ERS1)	forward GAGCTTATTGACGTTGTTGCAG reverse AGCATGGATTGCCTTCTCTG	AF092088
elongation factor 1-α (EF1)	forward ATTGACAAGCGTGTGATTGAGC reverse TCCACAAGGCAATATCAATGGTA	AY498567

Figure 1. Peel and flesh color of 'Comune' and 'Tardivo' clementines during fruit development and ripening.

To enable statistical analysis, real time RT-PCR experiments were performed with RNAs isolated from flavedo bulks (three for each genotype), and each real-time PCR sample was run in triplicate.

Ethylene Treatments. A preliminary assay was performed to analyze the effect of exogenous ethylene on peel coloration. Five 'Comune' and 'Tardivo' fruits were harvested at the end of November (during color-break stage of 'Comune') and dipped in 200 and 500 mg/L Ethephon (Ethrel, Bayer CropScience, Germany) solutions. Control fruits of both varieties were dipped in distilled water without Ethrel.

RESULTS

Evolution of the Qualitative Features of 'Comune' and 'Tardivo' during Fruit Development. Color change was the most marked difference between 'Comune' and 'Tardivo clementines. 'Comune' started the color-break in November, whereas 'Tardivo' began in December. They reached their respective orange peel colors in December and January (**Figure 1**), but the latter cultivar was less deeply colored than 'Comune'. 'Tardivo' showed juice acidity and TSS levels comparable to those of 'Comune' (**Figure 2**). These results indicated that 'Tardivo' was not a mutant modifying all of the ripening process, as internal maturity proceeds at the same rate as its parent, but showing a different rate of external fruit pigmentation. At harvest time (January for 'Comune' and February for 'Tardivo'), 'Tardivo' fruits showed similar diameter and slightly higher weight compared to 'Comune' (**Figure 3**). Concerning the clementine ripening parameters, the tendency of the fruit to reduce its weight and size was due to overmaturation, which led to a reduction of juice content as stated by Damigella and Continella (*22*). Juice reduction may have led to an overestimation of TSS and acidity during the last measurements.

Total Carotenoid Content and Carotenoid Profiles during Fruit Maturation. Quantifying total carotenoid in flavedos revealed differences in the genotypes from the early stages of development. The levels of 'Comune' and 'Tardivo' quickly declined between

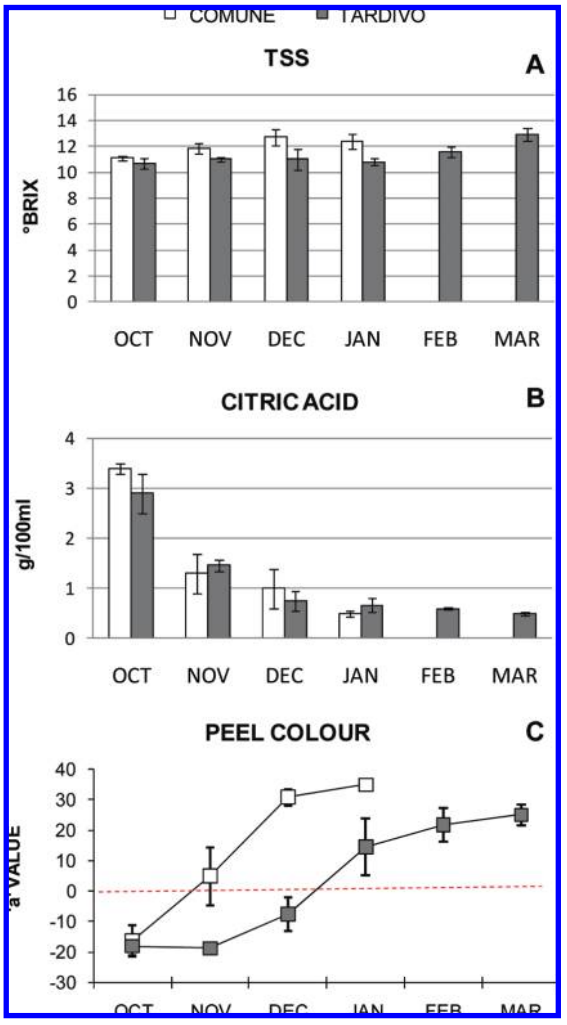

Figure 2. Physiological measurements performed on 'Comune' and 'Tardivo' clementine fruits, sampled on every first day of the month: (**A**) total soluble solids (TSS) in fruit juice; (**B**) citric acid in fruit juice, quantified indirectly by measuring total acidity (titration with 0.1 N NaOH); (**C**) peel color during development and ripening. Data are means ± SD (n = 3).

October and November. However, 'Comune' showed an increase in December and January, whereas the levels of 'Tardivo' declined in December and remained somewhat constant in January and February, increasing only in March (**Figure 4A**).

HPLC analysis revealed the profiles of phytoene, α-carotene, β-carotene, β-cryptoxanthin, lutein, and violaxanthin at different developmental stages (**Figure 4**). Six additional chromatographic peaks did not correspond to any known carotenoids. The accumulation of colorless phytoene, synthesized in the first carotenoid biosynthesis step, was markedly different in the two varieties from the first sampling on. Moreover, phytoene accumulation in 'Comune' was almost 4 times higher than in the late-ripening variety (**Figure 4B**). With regard to the colored carotenoids (α-carotene, lutein, β-carotene, β-cryptoxanthin, violaxanthin), 'Tardivo' generally showed delayed accumulation (**Figure 4C−G**). In general, accumulation trends reflected those found in clementine by Alos et al. (*4*), with a decrease in α- and β-carotene and lutein and an increase in cryptoxanthin and violaxanthin.

Analysis of Carotenoid Biosynthetic Genes. Phytoene synthase (*PSY1*) transcript levels in the 'Comune' and 'Tardivo' flavedos were similar in the early developmental stages, although following different patterns from November onward (**Figure 5A**). Specifically,

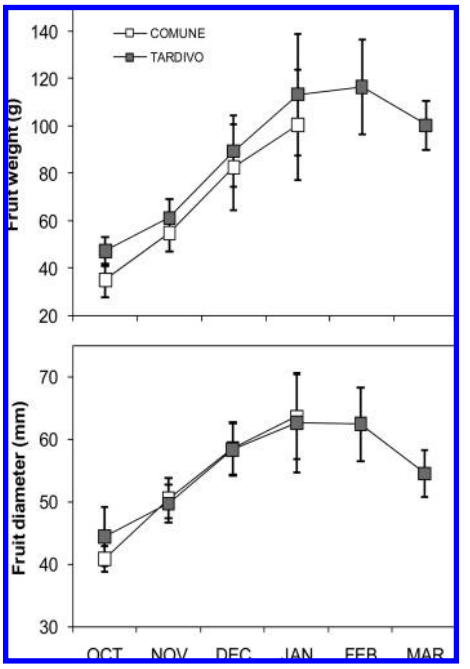

Figure 3. Fruit weight and diameter of 'Comune' and 'Tardivo' clementines measured every first day of the month during development and ripening. Data are means ± SD (n = 3).

only in 'Comune' did *PSY1* transcript levels clearly increase as the fruit matured.

With regard to ζ-carotene desaturase (*ZDS*), the late ripening genotype reached the same expression levels of 'Comune' about 2 months later (**Figure 5B**). The gene expression pattern of lycopene β-cyclase (β-LCY) was generally higher in 'Comune' from color-break (November) to the latest phase of fruit development (**Figure 5C**). The expression level of violaxanthin de-epoxidase (VDE) was similar for the two genotypes during the entire ripening period except in December when 'Comune' reached rind full color (**Figure 5D**). A general increase in carotenogenic gene expression was observed during fruit development.

Chlorophyllase Expression. Differences in the mRNA levels of chlorophyllase (*CHLASE1*) were detected in the flavedo of 'Comune' and its natural mutation 'Tardivo' during fruit development. The greatest chrorophyllase expression was observed in 'Comune' at color-break. During the other maturation phases no significant differences were observed (**Figure 6**).

Ethylene Biosynthesis and Perception. Differential expression of genes involved in the biosynthesis and perception of ethylene during fruit development and ripening in the two genotypes was observed. Two members of the ACC synthase gene family (*ACS1* and *ACS2*) and ACC oxidase (*ACO1*) were analyzed (*14*). Transcript levels in 'Comune' and 'Tardivo' were the same in October when peel color was fully green. A significant differential expression of ethylene genes (**Figure 7A−C**) was observed during November (when color-break started in 'Comune', whereas 'Tardivo' peel was still fully green) and December, but the expression levels were similar in January (when the rind reached full orange). The ethylene receptors analyzed belong to the ETR1-like subfamily, consisting of ETR1 and ERS1 (*14*), localized in the plasma membrane where ethylene binding occurs. Significant differences were observed in ethylene receptor *ETR1* and *ERS1* gene expression in November when transcript levels in 'Tardivo' were 2 and 3 times higher compared to those in 'Comune'(**Figure 7D**,**E**).

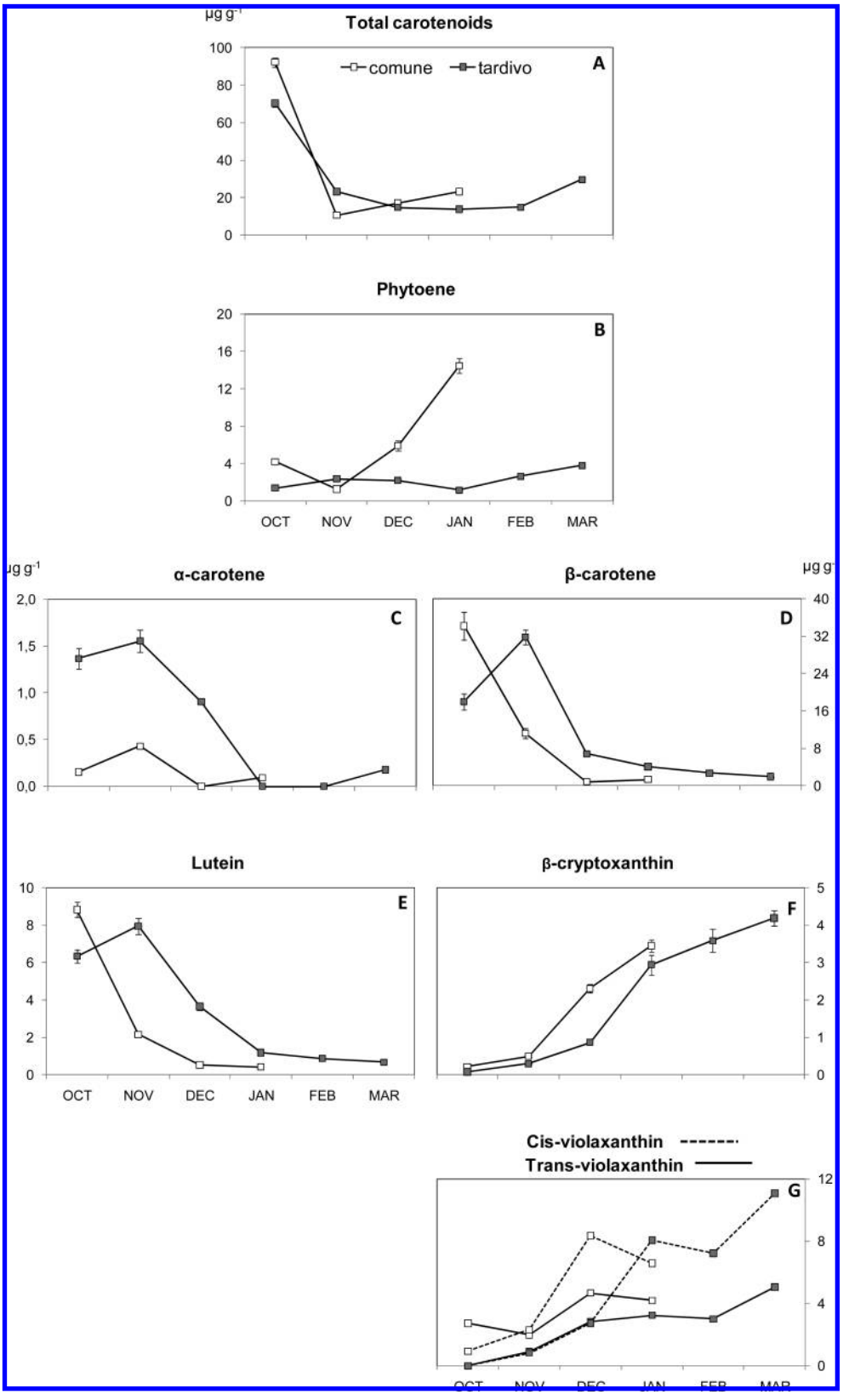

Figure 4. Carotenoids (μg g^{-1}) in the flavedo of 'Comune' and 'Tardivo' clementines during fruit development and ripening. Data are means ± SD (n = 3).

To assess whether the two genotypes could have different responses to ethylene, we performed a preliminary assay consisting of the exogenous application of ethylene (500 mg/L) in fruits harvested in November ('Comune' color-break). The assay showed full 'Comune' degreening after 4 days, but just a weak color change in 'Tardivo' (**Figure 8**).

DISCUSSION

Several citrus species and varieties have been characterized to investigate the physiological and molecular basis of ripening (*3*−*9*, *31*−*35*). In our study, 'Comune' clementine and one of its late-ripening mutations, 'Tardivo', were characterized through morphological, biochemical, and molecular analyses, to search

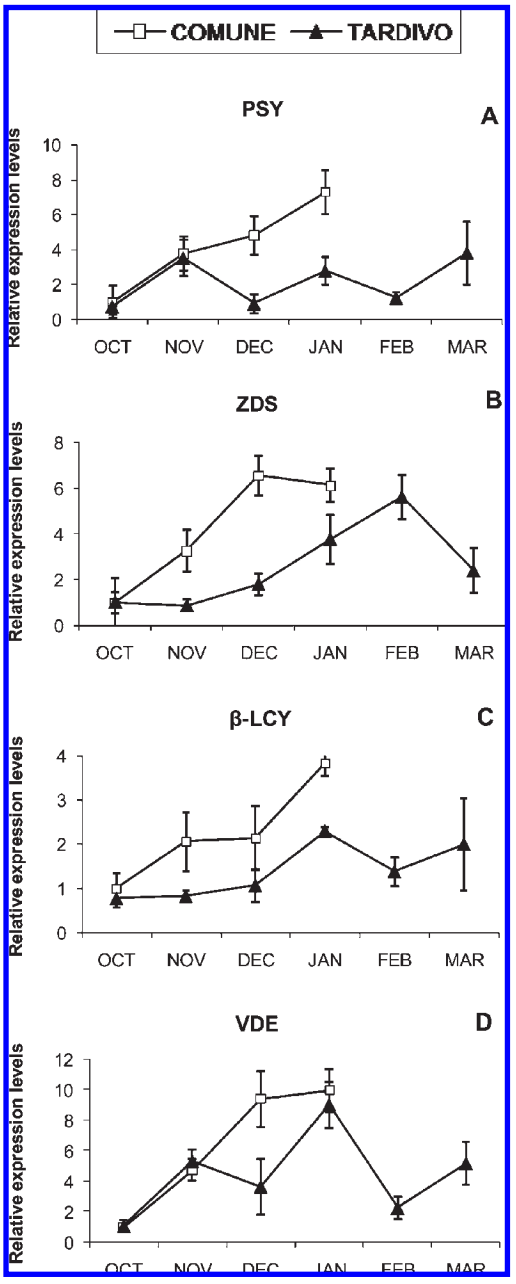

Figure 5. Analysis of relative transcript levels of genes involved in the carotenoid biosynthetic pathway, in the flavedo of 'Comune' and 'Tardivo' clementines: (**A**) phytoene synthase; (**B**) ζ-carotene desaturase; (**C**) β-lycopene cyclase; (**D**) violaxanthin de-epoxidase. Data are means ± SD (n = 3).

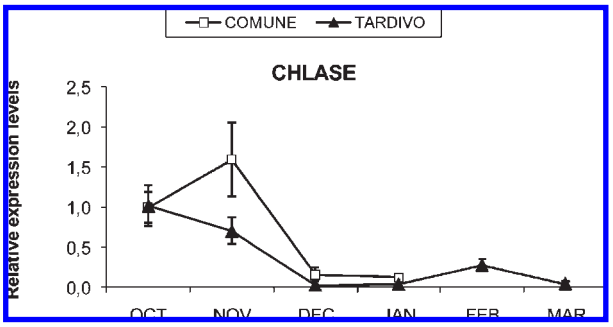

Figure 6. Analysis of relative transcript levels of the chlorophyllase gene in the flavedo of 'Comune' and 'Tardivo' clementines, sampled every first day of the month. Data are means ± SD (n = 3).

for differences affecting delayed maturation. 'Comune' started color-break during November, 'Tardivo' in December. They reached full orange in December and January, respectively (**Figure 1**). Juice acidity and TSS patterns (**Figure 2**), as well as weight and diameter, were not affected by the mutation; however, peel coloration was delayed, confirmed by HPLC analysis revealing quantitative differences in carotenoid accumulation.

Reports on carotenoid profiles and carotenoid gene expression patterns indicated that maturation and carotenoid accumulation are strongly dependent on genotype and that alterations leading to phenotype differences probably have a different nature. It seems that mutations leading to differences in ripening period and carotenoid profile probably affect different metabolic pathways. It is documented that in citrus, as in other species, the accumulation of carotenoid is regulated by the transcriptional activation of carotenoid biosynthetic genes (*3*). Our study agrees with previous studies, because carotenoid biosynthetic genes were regulated at transcription level. In particular, Kato et al. (*3*), as well as Rodrigo et al. (*5*), Fanciullino et al. (*9*), and Ikoma et al. (*36*), indicated that, during fruit ripening, the rise in the expression levels of *PSY*, *ZDS*, *β-LCY*, and other genes involved in carotenogenesis led to massive accumulation of β,β-xanthophylls, responsible for peel coloration.

In our case, a difference in carotenogenesis was observed at the biochemical and molecular levels. The main difference between the two varieties was related to different transcriptional *PSY* activity and phytoene accumulation. A previous work has shown that *PSY1* plays an important role in carotenoid accumulation (*36*), and it was thought to be the rate-limiting step in carotenoid biosynthesis. Studies on *PSY* expression have been conducted on *Citrus* varieties, and those reports clearly indicated that transcripts increased during fruit development and ripening (*3*, *5*, *36*, *37*). We observed similar behavior in 'Comune', whereas in 'Tardivo' *PSY* was generally down-regulated and did not reach the 'Comune' levels. These expression patterns correlate well with the phytoene quantification obtained by HPLC analysis, where full-colored 'Comune' accumulated 4 times more phytoene than full-colored 'Tardivo'. These results demonstrated that the mutation in 'Tardivo' influenced the transcriptional activation of *PSY*, a key step in carotenoid biosynthesis.

HPLC profiles from 'Tardivo' fruits showed delayed accumulations of α-carotene, β-carotene, lutein, and β,β-xanthophylls (β-cryptoxanthin and violaxanthin). However, at full coloration, both genotypes reached similar accumulation levels of these carotenoids. The delay in carotenogenesis was also revealed by real-time PCR analysis of *ZDS*, *β-LCY*, and *VDE*.

Another evident difference between the fruits of 'Tardivo' and 'Comune' was related to chlorophyllase gene expression. The *CHLASE1* gene encodes an active chorophyllase enzyme, which catalyzes the hydrolysis of chlorophyll (CHL) into phytol and represents the rate-limiting step in CHL catabolism. Transcriptional analysis of *CHLASE1* showed a difference between the two genotypes. *CHLASE1* expression in 'Comune' peel increased at color-break to gradually decrease afterward. Our results appear to contrast slightly with those of Jacob-Wilk et al. (*11*) for 'Valencia' orange peel, who found that *CHLASE1* expression was low and constitutive during natural fruit development without a significant increase during color-break and ripening. However, the same study and another (*20*) showed that ethylene treatment induced an increase in *chlorophyllase* transcript at all stages of development and accelerated chlorophyll breakdown. In particular, an enhanced response to ethylene treatment was observed during the time of natural color-break. Other papers

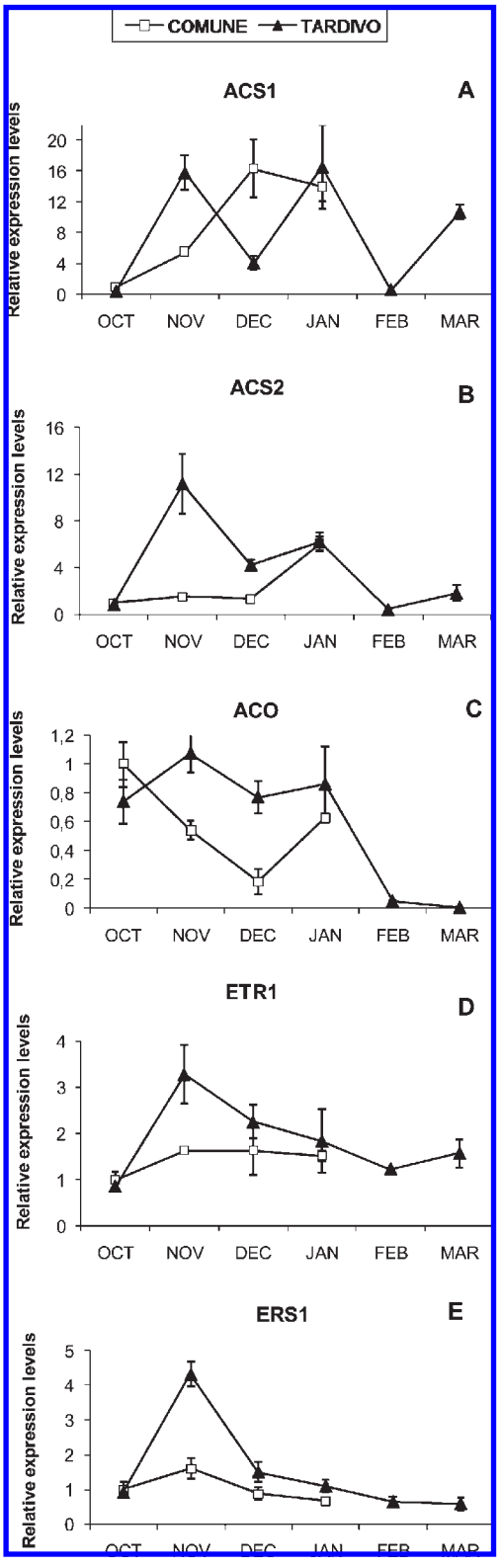

Figure 7. Analysis of relative transcript levels of genes involved in ethylene synthesis (**A**−**C**) and regulating ethylene response (**D**, **E**) in the flavedo of 'Comune' and 'Tardivo' clementines: (**A**) 1-aminocyclopropane-1-carboxylase (ACC) synthase 1; (**B**) ACC synthase 2; (**C**) ACC oxidase, (**D**) ethylene receptor ETR1; (**E**) ethylene receptor ERS1. Data are means ± SD ($n = 3$).

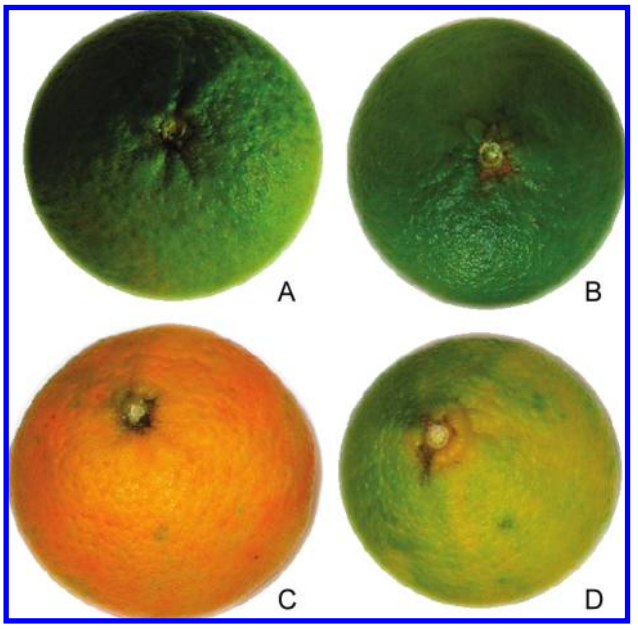

Figure 8. Altered responses of fruits harvested in November ('Comune' color-break) to the exogenous application of ethylene (500 ppm): (**A**, **B**) untreated fruits of 'Comune' and 'Tardivo' clementines; (**C**, **D**) fruits of 'Comune' and 'Tardivo' clementines 4 days after hormone application.

have indicated post-translational regulation of *CHLASE1* activity, although parallel transcriptional regulation (*38*, *39*) is hypothesized. Our data showed a transient increase in *CHLASE1* expression during 'Comune' color-break, whereas in 'Tardivo' there was lower expression.

Ethylene is likely involved in the regulation of carotenoid biosynthetic genes in both climateric and nonclimateric fruits (*21*, *40*−*42*). Ethylene is synthesized from *S*-adenosylmethionine via 1-aminocyclopropane-1-carboxylate (ACC). The enzymes catalyzing the two reactions in this pathway are ACC synthase and ACC oxidase. *ACC* synthase gene expression is differentially and closely regulated by various developmental, environmental, and hormonal signals (*43*). The production of ethylene is highly regulated, a key point involving the control of active ACS protein levels (*44*). Positive and negative feedback regulation of ethylene biosynthesis has been reported in different plant species (*45*−*47*). ACS proteins can heterodimerize and form active enzymes. The presence of various ACS isoforms, combined with distinct expression patterns, influences the optimization of expression and the stability of ACS enzymes regulating ethylene production (*44*).

Because delay in ripening and peel coloration might be caused by a perturbation of ethylene biosynthesis and perception (*48*, *49*), we decided to evaluate the transcriptional activity of genes involved in the ethylene pathway. Moreover, we performed a preliminary assay to evaluate the influence of exogenous ethylene treatments on peel degreening during 'Comune' color-break. Our results showed clear differences in the expression of ethylene biosynthetic genes. The greatest difference between the two genotype mRNA amounts of *ACS1*, *ACS2*, and *ACO1* was observed between November and December. These data suggest that these two months represent (when 'Comune' was at the color-break stage and 'Tardivo' was still green) the key period when differential gene expression determined a change in fruit coloration dynamics. The differences between the two genotypes were also revealed by differential response to ethylene treatment, which caused full degreening for 'Comune' peel and just a slight color change in 'Tardivo'.

To assess whether 'Tardivo' had higher ethylene levels or the mRNA levels of the biosynthetic genes in 'Comune' were down-regulated due to a negative feedback mechanism, we tried to measure endogenous ethylene levels of fruit one day after harvesting

throughout the ripening period, but the levels detected were extremely low, so varietal behavior was indistinguishable. Only one study analyzed ethylene evolution in citrus fruits (*14*). This study showed an extremely low amount of endogenous hormone produced during fruit development (about 0.05 nL g^{-1} h^{-1}), whereas ethylene production increased dramatically in detached fruitlets 3−5 days after harvesting (25−70 nL g^{-1} h^{-1}). However, this autocatalytic behavior in detached fruit may not be linked to the ripening process, likely being the result of stress during detachment or related to abscission (*49*).

In addition to ethylene biosynthetic genes, we analyzed the expression level of two ethylene receptors (ETR1 and ERS1) (*14*). Models of ethylene signal transduction indicated that ethylene receptor mRNA levels negatively correlate with ethylene sensitivity (*44*, *50*−*52*). Both *ERS1* and *ETR1* genes, involved in ethylene signal transduction, showed differential expression patterns in the genotypes. In particular, the mRNA levels were somewhat constitutive in 'Comune', whereas in 'Tardivo' they peaked in November. The differences related to *ACS1*, *ACS2*, *ACO1*, *ERS1*, and *ETR1* gene expression, as well as the different response to exogenous ethylene treatments, suggested an altered sensitivity of 'Tardivo' to ethylene, which in turn may have delayed carotenogenesis. Similar behavior occurred in tomato fruits (*48*), in which the down-regulation of an *ETR* gene caused only a slight change in ripening period, that is, slower carotenoid accumulation.

In conclusion, the differences in ripening period might have been influenced by an alteration in 'Tardivo' ethylene signaling compared to 'Comune'. This perturbed sensitivity could have influenced carotenoid biosynthesis and accumulation, especially PSY expression and phytoene accumulation. How flavedo cells perceive and respond to low levels of ethylene in the fruit is still unknown, but the results suggest a link between ethylene perception and biosynthesis, as shown in other systems. However, this hypothesis requires confirmation.

The link between ethylene and chlorophyll degradation deserves further research because in citrus the regulation of chlorophyll metabolism is a major component of the resistance to degreening. We have shown that *CHLASE1* is down-regulated in 'Tardivo' clementine, whereas it has been found that *39B3*, another color delay mutant of clementine, has a deletion including genes of two multifunctional chloroplastic proteases (*35*). Interestingly, these proteases and *CHLASE1* are major elements of the chlorophyll catabolism participating in the conversion of chlorophyll *a* to chlorophyll *b* and in the hydrolysis of chlorophyll *a*, respectively. It is also worth mentioning that the *nan* mutant of orange that is an ethylene responsive mutant does not break down chlorophylls (*34*), suggesting a mutation upstream of ethylene signaling or an independent pathway. Moreover, the analysis of flavedo transcriptome may help to identify regulatory genes that may affect fruit development and ripening. For this reason, we are performing a cDNA-based screening technique to search for differentially expressed genes in the two genotypes.

ABBREVIATIONS USED

ACC, 1-aminocyclopropane-1-carboxylate; ACS1, ACS2, ACC synthase; ACO1, ACC oxidase; ERS1, ETR1, ethylene receptors.

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