Scientia Horticulturae 254 (2019) 208–214

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Low-temperature storage regulates the expression of genes related to peel pigments of grapefruit

Emmanuel Aispuro-Hernández[a], Araceli Minerva Vera-Guzmán[b], Irasema Vargas-Arispuro[a], Miguel Ángel Martínez-Téllez[a,*]

[a] Centro de Investigación en Alimentación y Desarrollo, A. C. (CIAD), Centro Público de Investigación del Consejo Nacional de Ciencia y Tecnología (CONACyT), Carretera Gustavo Enrique Astiazarán Rosas # 46, Col. La Victoria, Hermosillo, 83304 Sonora, Mexico
[b] Instituto Politécnico Nacional, CIIDIR Unidad Oaxaca, Hornos # 1003, Santa Cruz Xoxocotlán, 71230 Oaxaca, Mexico

ARTICLE INFO

Keywords:
β-Carotene
Carotenoid biosynthetic pathway
Chlorophyll
Citrus paradisi
Cold storage
Lycopene

ABSTRACT

Grapefruit fruit (*Citrus paradisi* Macfad) are sensitive to postharvest temperature variations. In red grapefruit cultivars, peel color is a characteristic and important quality parameter that may undergo changes during storage due to alterations in the content of pigments. So far, it is not clear if the postharvest peel color variations in grapefruit are regulated at the transcriptional level due to storage conditions. The objective of this work was to investigate the effect of postharvest storage temperature (2 and 13 °C) on the relationships between peel color, chlorophyll (Chl) and carotenoid contents and the expression of genes related to the synthesis and degradation of these pigments in the flavedo of 'Rio Red' grapefruit grown in Northern Mexico. Fruit were harvested at breaker stage with a maturity index of 4.8 and stored for 42 days at 2 and 13 °C. Pigments, transcript levels, color index (CI) and ethylene production were periodically evaluated through storage. Pearson's correlations between CI and Chl, lycopene, and β-carotene contents were evaluated. Storage at 13 °C increased lycopene content and CI and decreased Chl content. In fruit stored at 2 °C, the CI and content of β-carotenes remained almost unchanged. In contrast, the lycopene content increased at this temperature, but to a lesser extent than at 13 °C. The ANOVA showed the expression of ζ-*carotene desaturase* (*ZDS*), two types of *lycopene cyclases* (carotenoid biosynthesis pathway genes) and the *pheophorbide a oxygenase* gene (involved in Chl breakdown), was significantly influenced by the storage time-temperature interaction. The expression of the *ZDS* gene was significantly higher in fruit stored at 13 °C than in fruit stored at 2 °C, suggesting that lycopene biosynthesis is transcriptionally regulated by storage temperature. The lycopene levels showed a positive relation with the *ZDS* transcript levels and CI, indicating this pigment is implicated in the red coloration of the 'Rio Red' grapefruit flavedo during postharvest.

1. Introduction

Grapefruit (*C. paradisi*) is a citrus fruit with worldwide commercial importance and outstanding emerging properties related to its bioactive compounds. In addition to their flavor, red grapefruit cultivars are of particular interest due to their reddish-pink pulp and skin tonalities. The external color is one of the main determinants of fruit quality for consumers' acceptance (Arpaia and Kader, 1999). Citrus fruit color diversity results from the synthesis and differential accumulation of chlorophylls (Chl) and carotenoids during maturation. Chl is the predominant pigment found in non-ripe grapefruit (Alquezar et al., 2013). During ripening of citrus fruit peel, the transition from chloroplasts into chromoplasts is characterized by complete Chl degradation, disassembly of thylakoid membranes, starch grain disappearance and gradual development of different sink structures associated with the accumulation of newly synthesized carotenoids (Lado et al., 2015a; Li and Yuan, 2013). The activation of Chl breakdown processes is concomitant to the synthesis of carotenoid pigments (Rodrigo et al., 2004).

Plant carotenoids are a large family of 40-carbon isoprenoid derived-compounds with highly conjugated polyene chains that confer attractive colors and contribute to the yellow, orange, pink and red tonalities (Alquezar et al., 2008). The main carotenoids found in the grapefruit flavedo (colored part of the rind) are phytoene, phytofluene, ζ-carotene, β-carotene, β-cryptoxanthin and 9-*cis*-violaxanthin, while red cultivars also contain varying amounts of lycopene. The red and pink color in grapefruit results from the differential accumulation of the carotenoids lycopene and the β-carotene, respectively associated with the red and orange color (Alquezar et al., 2013; Xu et al., 2006). The

* Corresponding author.
E-mail address: norawa@ciad.mx (M.Á. Martínez-Téllez).

https://doi.org/10.1016/j.scienta.2019.04.085
Received 21 February 2019; Received in revised form 26 April 2019; Accepted 29 April 2019
Available online 11 May 2019
0304-4238/

balance in the biosynthesis and accumulation of these carotenogenic pigments during ripening is endogenously regulated by the expression of the genes *phytoene synthase* (*PSY*), *phytoene desaturase* (*PDS*), ζ-*carotene desaturase* (*ZDS*) and *lycopene cyclases* (*LCY*) (Alquezar et al., 2008); lycopene is produced by ZDS after desaturation of ζ-carotene, which subsequently may or not be converted to α- or β-carotene by LCY (Alquezar et al., 2013).

Despite this complex endogenous regulation, external factors such as pre- and postharvest conditions may influence the speed of metabolic events like degradation or de novo synthesis of relevant pigments, which will impact the final fruit quality and storage life. Ethylene application during storage has been proven to improve grapefruit peel color by accelerating degreening in the flavedo, while the effect on the content of bioactive compounds has been controversial as some phytochemicals are increased but others are decreased (Chaudhary et al., 2015, 2012). Likewise, temperature is an environmental factor that substantially influences the presence of color-related compounds in citrus fruits; it regulates biochemical processes associated with the degradation of Chl and the synthesis of carotenoids and other pigments (Goldschmidt, 1988). In general, carotenoid biosynthesis in citrus fruits is sensitive to temperature variations (Matsumoto et al., 2009), with an optimum range between 15 and 25 °C (Wheaton and Stewart, 1973). Preharvest temperatures below 14 °C induce a faster degreening of grapefruit (Manera et al., 2013), while the postharvest storage temperature modulates the amount of phytochemicals accumulated in grapefruit pulp (Chaudhary et al., 2014) and orange peel (Carmona et al., 2012).

Cold storage is the most common technology used to extend shelf-life in citrus fruits. Although the recommended temperature for preserving the quality of grapefruit is between 12 and 14 °C (Ladaniya and Ladaniya, 2008), temperatures under 8–10 °C are frequently used during the required quarantine periods prior to exporting citrus fruits to fly-free zones in many countries (Biolatto et al., 2005). Low postharvest storage temperatures are known to delay degreening of citrus fruits (Ritenour et al., 2018), and recently the modulation in the expression of the Chl breakdown related genes *staygreen* (*Sgr*) and *pheophorbide a oxygenase* (*PaO*) by effect of storage temperatures was demonstrated in kiwi fruit (Gambi et al., 2018). An enhanced coloration in the flavedo of 'Navelina' orange was induced by a storage temperature of 12 °C, which increased the concentration of carotenoid pigments, whereas the color in fruit stored at 2 °C remained unchanged (Carmona et al., 2012). A storage temperature of 20 °C dramatically increased the total carotenoid content in 'Satsuma' mandarin due to a rise in the levels of β-cryptoxanthin and 9-*cis*-violaxanthin, whereas a slower carotenoid accumulation was observed at 5 °C (Matsumoto et al., 2009). Likewise, Carmona et al. (2012) and Matsumoto et al. (2009) found a temperature-dependent response in the gene expression of several carotenoid biosynthesis-related enzymes, particularly *ZDS* expression increased during storage at 12 °C in 'Navelina' orange and at 20 °C in 'Satsuma' mandarin fruits.

Although temperature influences Chl degradation and carotenoid biosynthesis in the flavedo of citrus fruit, the effect of this parameter on carotenoid accumulation in grapefruit, has only been focused on monitoring the levels of important phytochemicals in the pulp and flavedo (Lado et al., 2015b; Chaudhary et al., 2014). In particular, there are no reports about the effect of cold stress temperatures on the expression of genes related to degreening and synthesis of carotenoid pigments in the peel of grapefruit fruit during postharvest storage. Since 'Rio Red' grapefruit has characteristic attractive features, such as reddish-yellow skin, it can be used as a model cultivar to study the postharvest changes associated with peel color development in lycopene-rich citrus fruits. Therefore, the aim of this study was to evaluate the effect of storage temperature and time on physiological and biochemical parameters related to flavedo color development in 'Rio Red' grapefruit stored at low temperatures.

2. Materials and methods

2.1. Fruit material

A sample of 246 grapefruit (*C. paradisi* cv. Rio Red) fruit were harvested at the beginning of November 2013 from bearing trees from an experimental orchard located in La Costa of Hermosillo, Sonora, Mexico, (lat. 28°45′0.98″N, long. 111°27′26.17″W), which is a warm desert region with an annual average temperature of 23 °C and an altitude of 54 masl. The fruit were transferred to the laboratory, visually inspected to be free of damage and defects, selected, randomized, washed and disinfected with sodium hypochlorite solution (Ballester and Lafuente, 2017). The maturity index was calculated as the SST/TA ratio, as described by Vera-Guzman et al. (2017).

2.2. Experimental

Considering the high susceptibility of grapefruits to quarantine cold treatments, the parameters were evaluated in fruit stored either at 2 °C (chilling temperature) or at 13 °C (recommended storage temperature to delay fruit senescence). The disinfected fruit were divided into six independent lots, each containing 41 grapefruits: three lots were stored at 2 °C and three at 13 °C, with 90 to 95% relative humidity (RH) for 0, 21, and 42 days and the data were collected following each storage period. Later, 20 fruit per replicate were used to monitor color index (CI) and weight loss, and three fruit were sampled per replicate to evaluate ethylene production. The flavedo was collected from four fruit per replicate, homogenized, and used to determine the total Chl, lycopene and β-carotene contents as well as the transcript levels of the *PSY*, *PDS*, *ZDS,* β-*LCY* and ε-*LCY* genes; implicated in the carotenoid biosynthesis pathway, and the *Sgr* and *PaO* genes; associated with Chl breakdown processes.

2.3. Analysis of the total Chl, lycopene and β-carotene contents

The total Chl (a + b), lycopene and β-carotene contents were extracted according to the method reported by Davis et al. (2003). The absorbance of the extracts was measured with the UV biospec-1601 spectrophotometer (Shimadzu®, Kyoto, Japan) at 663 and 645 nm for Chl a and b, 505 nm for lycopene and 453 nm for β-carotene. The pigments contents were calculated using the following equations described by Nagata and Yamashita (1992):

$$\text{Chl a (mg } 100\,\text{m L}^{-1}) = 0.999A_{663} - 0.0989A_{645}$$

$$\text{Chl b (mg } 100\,\text{m L}^{-1}) = -0.328A_{663} + 1.77A_{645}$$

$$\text{Lycopene (mg } 100\,\text{m L}^{-1}) = -0.0458A_{663} + 0.204\,A_{645} + 0.372A_{505} - 0.0806A_{453}$$

$$\beta\text{-carotene (mg } 100\,\text{m L}^{-1}) = 0.216\text{A}_{663} - 1.22\quad \text{A}_{645} - 0.304\text{A}_{505} + 0.452\text{A}_{453}$$

where $A_{663,}$ $A_{645,}$ $A_{505,}$ and A_{453} are the absorbances at 663 nm, 645 nm, 505 nm, and 453 nm, respectively. The results were expressed as μg g^{-1} FW.

2.4. Expression levels of genes related to the synthesis and degradation of pigments

Total RNA was isolated from 0.1 g of frozen grapefruit flavedo tissue using the protocols reported by Reid et al. (2006) with some modifications. The isolated RNA was treated with the DNA-free kit (Ambion, USA) to remove the genomic DNA. The quality and integrity of the total RNA was evaluated by the absorbance ratios A260/A280 and A260/A230 using a NanoDrop 2000 (Thermo Scientific NanoDrop, USA) and by electrophoresis on 1% denaturing agarose gel. Total RNA (3 μg) was reverse transcribed using the SuperScript II Reverse Transcriptase kit

Table 1
Primers used in semi-quantitative RT-PCR analysis of gene expression.

Gene	Primer sequence (5′ to 3′)	GenBank accession number
Phytoene synthase (*PSY*)	Fw-GATGTTGGAGAGGATGCCCG Rv- CATGCTGCATACCAACAATTCC	XM_006481880
Phytoene desaturase (*PDS*)	Fw-GTGAACCGATGGGTCAGAGC Rv-CCGGCCAAGTCAGCATTTCA	AF364515
ζ-Carotene desaturase (*ZDS*)	Fw-CGATCCTTACATGCCCTTAC Rv-AGGTCCCTCACGGTACAAAG	AF372617
Lycopene β-cyclase (*β-LCY*)	Fw-CTACTTGGTCTGGTGCCGTT Rv-CGGGTGCTGTTCTACCTCAG	JF907401
Lycopene ε-cyclase (*ε-LCY*)	Fw-AAACCTCGCATTTGGTGCTG Rv-AAGGAAACCGTGCCACATCC	AF486650
Staygreen (*Sgr*)	Fw-GCAAGATTATTCGGACCAGCCA Rv-TGGGTAAGTGTATAAGTCCTTGGC	MF945620; XM_006477286; XM_006440432
Pheophorbide a oxygenase (*PaO*)	Fw-TGGTCTTTTGACGGGTGTGG Rv-CGTGCCATCTAGGAACCACC	XM_006487933; XM_006424372
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	Fw-TTGTGATCTCCGCCCCTAGC Rv-AGCAAGAGGAGCTAGGCAGT	MH170284

(Invitrogen, USA), according to the manufacturer's recommendations. cDNAs were used as the template for the PCR reactions with the GoTaq® Flexi DNA Polymerase kit (Promega, Madison, WI, USA) and the primers listed in Table 1, which were designed based on either specific *C. paradisi* sequences or highly conserved sequences from citrus fruits in the GenBank of NCBI. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as a reference gene. The PCR products were sequenced at Macrogen Inc. (Seoul, Korea) to confirm their identity and the relative expression levels were calculated from the signal intensity of the electrophoresed bands on 1% agarose gel that had been stained with Gel-Red (Biotium, Hayward, CA, USA) and visualized under UV light. Data was double-normalized, first against the expression levels of GAPDH and then to values at day zero. The results were presented as the means of three biological replicates ± SE.

2.5. Color index

Peel color was analyzed by using a Minolta CR-300 colorimeter (Konica Minolta Inc., USA) at four locations around the equatorial plane of 20 fruit per treatment. Values were calculated as CI = (1000 x a/L x b). In this formula, L is luminosity (0 = black to 100 = white), a = green (−) to red (+) and b = blue (−) to yellow (+) (Moscoso-Ramírez and Palou, 2014).

2.6. Ethylene analyses

Three fruit per replicate were incubated in sealed glass containers at 24 °C for 4 h. One mL gas sample was withdrawn from the headspace of the container and ethylene production was quantified by gas chromatography (Varian Star 3400) with a Hayesep N column using nitrogen as carrier gas and a flame ionization detector (FID) as described by Vera-Guzman et al. (2017). Ethylene standards were obtained from Praxair (Hermosillo, México). The results were expressed as μL C_2H_4 kg^{-1} h^{-1}.

2.7. Statistical analysis

The data were subjected to analysis of variance to evaluate the effects of temperature, storage time and the temperature-storage time interaction on the pigment contents, CI and ethylene production, as well as the relative gene expression levels, using NCSS version 12 data analysis software (NCSS, LLC, Kaysville, UT, USA). A mean comparison using Tukey's test ($P < 0.05$) was performed to determine the differences within the evaluated factors. The relationship between the pigment compounds and CI was assessed using Pearson's correlation analysis.

3. Results and discussion

The mean value of the maturity index in 'Rio Red' grapefruits at harvest was 4.8 ± 0.6, which is near to the recommended by Arpaia and Kader (1999) and similar to reported values for 'Ruby Red' grapefruit (Ramin and Alirezanezhad, 2005) and 'Red Blush' grapefruit (Muhtaseb, 2007). Fruit showed an average weight of 339 ± 23 g at harvest that remained unchanged during storage at 2 and 13 °C with 90–95% RH for 42 days, indicating that these conditions were adequate to prevent fruit dehydration.

3.1. Effect of storage temperature on rind pigment compounds and color

The effect of the postharvest temperature on Chl, lycopene, β-carotene and color was investigated during a period of 0, 21 and 42 days at 2 °C or 13 °C. The ANOVA detected significant differences among the temperature, storage time and the temperature-storage time interaction for all evaluated pigments and CI.

Fig. 1A shows the changes of flavedo Chl content on grapefruit stored at 2 °C and 13 °C. The total Chl content decreased (93%) with storage time at 13 °C. Chl degradation was lower for fruit stored at 2 °C (69%). Temperature can accelerate or delay Chl degradation and differentiation of chloroplasts into chromoplasts during ripening (Li and Yuan, 2013). This dynamic process is mediated by a set of catabolic enzymes that first release the porphyrin ring from the thylakoid-anchored phytol chain, then dechelate magnesium and hydrolyze the ring (Shimoda et al., 2016). In citrus fruits, heat treatment delays Chl degradation by suppressing the activity of Chl-degrading enzymes such as chlorophyllases, Mg-dechelatase and oxidases during postharvest storage (Kaewsuksaeng et al., 2015), which along with the results observed in our work, may likely indicate Chl breakdown pathway comprise temperature-sensitive components. In *Arabidopsis* the *Sgr* gene codes for a Mg-dechelatase enzyme, which is also necessary for destabilizing the light-harvesting complex; steps that occur previous to the hydrolysis of the porphyrin ring by the PaO enzyme, during Chl recycling and degradation processes (Shimoda et al., 2016).

The flavedo color in ripe grapefruit is mainly determined by the content and ratio of lycopene and β-carotene (Xu et al., 2006; Alquezar et al., 2008). The effect of storage time and temperature on lycopene content in the flavedo of 'Rio Red' grapefruit stored at 2 and 13 °C during 42 days is presented in Fig. 1B. Lycopene increased during the storage period at both temperatures. At the beginning of the storage, there was a lycopene concentration of 13.4 μg g^{-1} of fresh flavedo tissue. A significant increment of 44% was noticed at day 21 of storage at 13 °C, which further increased up to 115% by day 42. A lower increment in lycopene content was observed in fruit stored at 2 °C, with an increase of 27% and 53% by days 21 and 42, respectively; which likely indicates lycopene synthesis occurs as part of the maturation process, but also that temperature may regulate its accumulation in red grapefruit cultivars. Fruit weight loss was not significative during the storage period, therefore the observed lycopene increments were mainly due to de novo biosynthesis and not by the concentration of the previously synthesized pigment. These results suggest lycopene accumulation in 'Rio Red' grapefruit to be temperature sensitive, and that some physiological processes influencing quality aspects, like fruit color, may follow a different behavior according to the postharvest condition. Temperature is one of the main regulating factors for carotenoid metabolism in citrus fruits (Matsumoto et al., 2009; Wheaton and Stewart, 1973). Such regulation may rely on the expression patterns of some temperature-sensitive transcription factors or structural

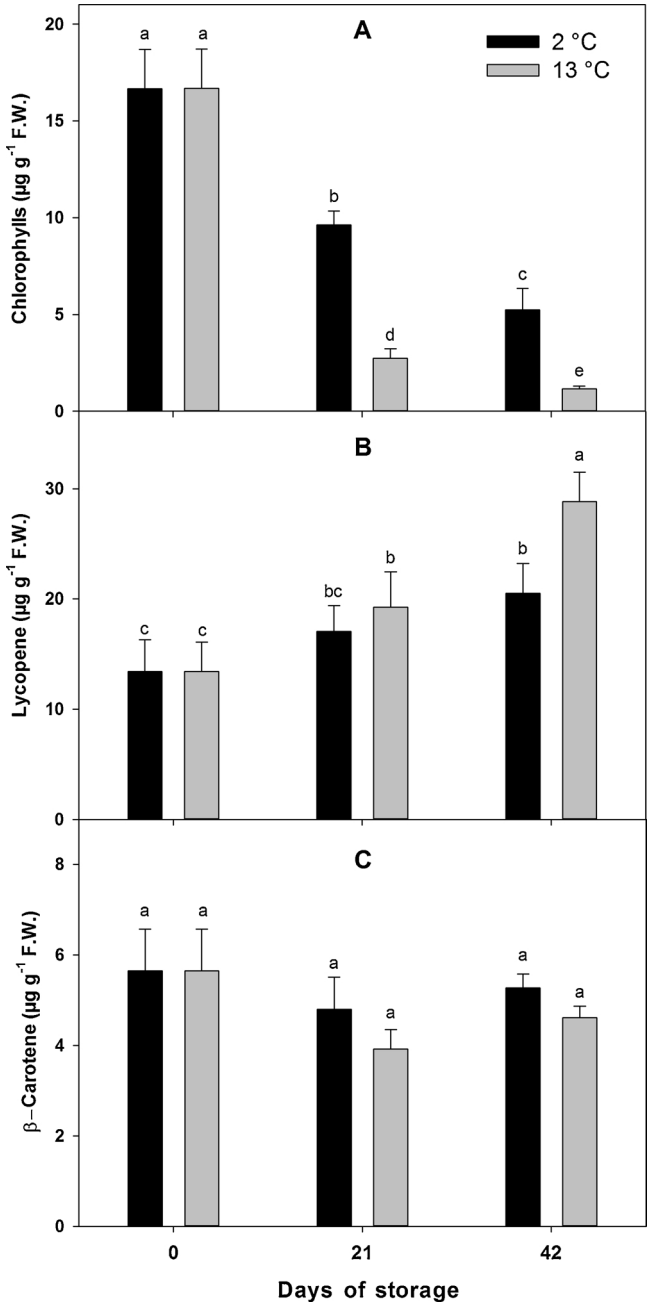

Fig. 1. Chlorophyll (A), lycopene (B) and β-carotene (C) contents in the flavedo of 'Rio Red' grapefruit fruit stored at 2 °C and 13 °C. Each value is the mean of three replicate samples ± SD. Values with the different letter are significantly different according to Tukey test ($P \leq 0.05$).

genes such as *PSY*, *PDS*, *ZDS*, *LCY*, *β-carotene hydroxylase* and *zeaxanthin epoxidase*, involved in the synthesis of lycopene and other carotenoid pigments (Carmona et al., 2012; Tao et al., 2012).

On the other hand, there were no significant differences in the accumulation of β-carotene between fruit stored at 2 °C and 13 °C (Fig. 1C). These results suggest the effect of temperature on the carotenoid accumulation in the flavedo of 'Rio Red' grapefruit may be different according to the type of carotenoid. The biosynthesis of α- and β-carotene relies on the cyclization of lycopene by the enzymes lycopene epsilon- or beta-cyclases (ε-LCY and β-LCY, respectively) (Alquezar et al., 2013, 2009); therefore, reduced *LCY* expression during maturation may simultaneously reduce the synthesis of α- and β-carotene derivatives and favor the accumulation of lycopene.

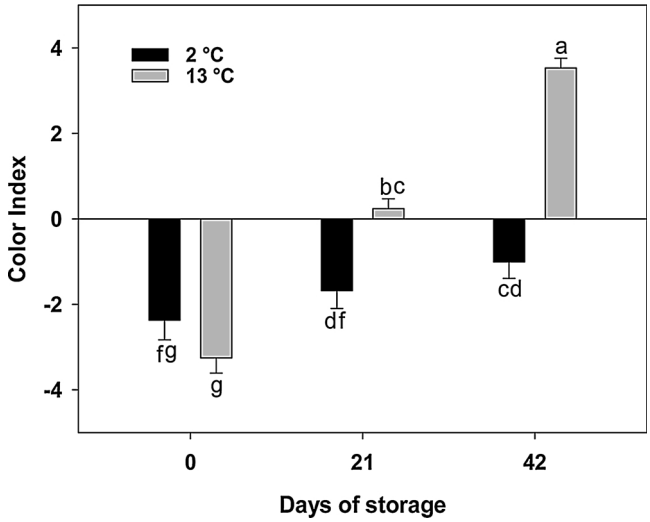

Fig. 2. Color Index of 'Rio Red' grapefruit fruit stored at 2 °C and 13 °C at days 0, 21 and 42. Each value is the mean of sixty fruit ± SE. Values with the different letter are significantly different according to Tukey test ($P \leq 0.05$).

The accumulation of lycopene in the grapefruit flavedo under storage conditions appears to be dependent on the temperature-time interaction. Lado et al. (2015b) observed an increased lycopene accumulation in 'Star Ruby' grapefruit flavedo after 56 days of storage at 12 °C. Meanwhile, Chaudhary et al. (2015) showed that lycopene levels increase in 'Rio Red' grapefruit juice vesicles after 14 days of storage at 11 °C. Our results presented these types of behaviors in the rind of 'Rio Red' grapefruit, where lycopene accumulation was sensitive to low storage temperatures, while β-carotene concentrations were not significantly affected, which agrees with results reported by Tao et al. (2012) and Chaudhary et al. (2015) for the red-fleshed 'Cara Cara' orange and the 'Rio Red' grapefruit, respectively.

3.2. Effect of the temperature-storage time interaction on rind color

The effects of the storage temperature-time interaction on the CI of 'Rio Red' grapefruit are shown in Fig. 2. The flavedo CI in fruit stored at 2 °C increased over time, but at 13 °C, the response was exponential from 0 to 42 days. The peel of citrus fruit may undergo changes in color during postharvest storage in response to temperature, such as degreening (Chl degradation) (Fig. 1A) or the de novo synthesis of lycopene (Fig. 1B) and another carotenoids at temperatures that do not produce chilling stress (Carmona et al., 2012). At day 42, the flavedo of fruit stored at 2 °C had negative values for CI or near zero at the end of the storage period, indicating that greenish tones could have remained on the surface. However, fruit stored at 13 °C presented the highest CI values indicating that the intensity of yellowness or redness was affected by the temperature-storage time interactions. The grapefruit flavedo underwent earlier fruit maturation at 13 °C than at 2 °C, which may explain the low CI values. Therefore, storage temperature seems to be an important factor for color regulation in the flavedo of 'Rio Red' grapefruit. Similar responses to storage temperature were observed in 'Satsuma' mandarin (Matsumoto et al., 2009) and 'Valencia' oranges (Carmona et al., 2012). In this work, the CI, conveniently explained the fruit peel color changes during storage at 2 °C or 13 °C.

Table 2 shows the correlations between the pigments contents and the CI. Chl displayed significant negative correlations ($P < 0.01$) with CI, indicating that higher Chl content is associated with reduced brightness and visual expression of the color in the flavedo of 'Rio Red' grapefruit. Lycopene content showed a significant positive correlation ($P < 0.01$) r = 0.72 with CI in the flavedo tissue, indicating that fruit with a more reddish flavedo contain a higher lycopene concentration.

Table 2
Pearson's correlations between chlorophyll, lycopene and β-carotene contents and color index (CI) of grapefruit flavedo stored during 42 days.

Pigment	CI
Chlorophylls	−0.69*
Lycopene	0.72*
β-Carotene	−0.16ns

ns: non significant at $P > 0.05$.
* Significant at $P < 0.01$ (Student test).

Nevertheless, β-carotene levels did not significantly correlate with CI, which indicates flavedo color is not influenced by the variations in β-carotene. These relationships explain some of the main changes observed in the analyzed pigments and their contribution to the peel color of grapefruit fruit during postharvest.

3.3. Expression of carotenoid biosynthesis pathway genes in response to low temperature storage

Fig. 3A–D shows the effect of temperature and time of storage on the expression levels of carotenogenic genes involved in the conversion of geranyl diphosphate to the colored compounds lycopene, α-carotene and β-carotene. The expression levels of *PSY* significantly increased during time of storage (Fig. 3A), independently of storage temperature. *PDS* expression showed relatively steady levels that were not significantly affected by any of the evaluated factors or its interaction (Table 3). These findings indicate that the initial steps of the carotenoid biosynthetic pathway are not regulated at the transcriptional level by the time-temperature interaction. The main influence of storage time and temperature interaction occurred on the expression of genes arranged in the central part of the pathway, such as *ZDS*, β-*LCY* and ε-*LCY*, which are directly implicated in the synthesis of colored carotenoids.

The expression of ZDS determines the accumulation of lycopene in grapefruit flavedo and is particularly critical in the stage prior to lycopene formation (Alquezar et al., 2013). In this work, *ZDS* gene expression was sensitive to changes in temperature. At 13 °C, the relative *ZDS* gene expression was higher than at 2 °C, while a similar effect was observed in fruit stored for 42 days compared to 21 days (Fig. 3B). The highest *ZDS* expression levels were found by day 42 of storage at 13 °C, and this consistent response was positively associated with the highest lycopene accumulation observed at day 42 (Fig. 1B).

Constant β-*LCY* expression levels were found during storage at 13 °C, while a drastic decrease was notorious by day 42 at 2 °C (Fig. 3D). At least two variants of the β-*LCY* have been characterized in *C. paradisi*. In the red grapefruit cv. Star Ruby, the preferential transcription of a non-functional chromoplast-specific β-*LCY* (β-*LCY2* gene) is responsible for the red coloration of the peel during fruit ripening (Alquezar et al., 2009). Since the expression of an inactive isozyme would not allow the enzymatic reduction of lycopene levels, the β-*LCY2*

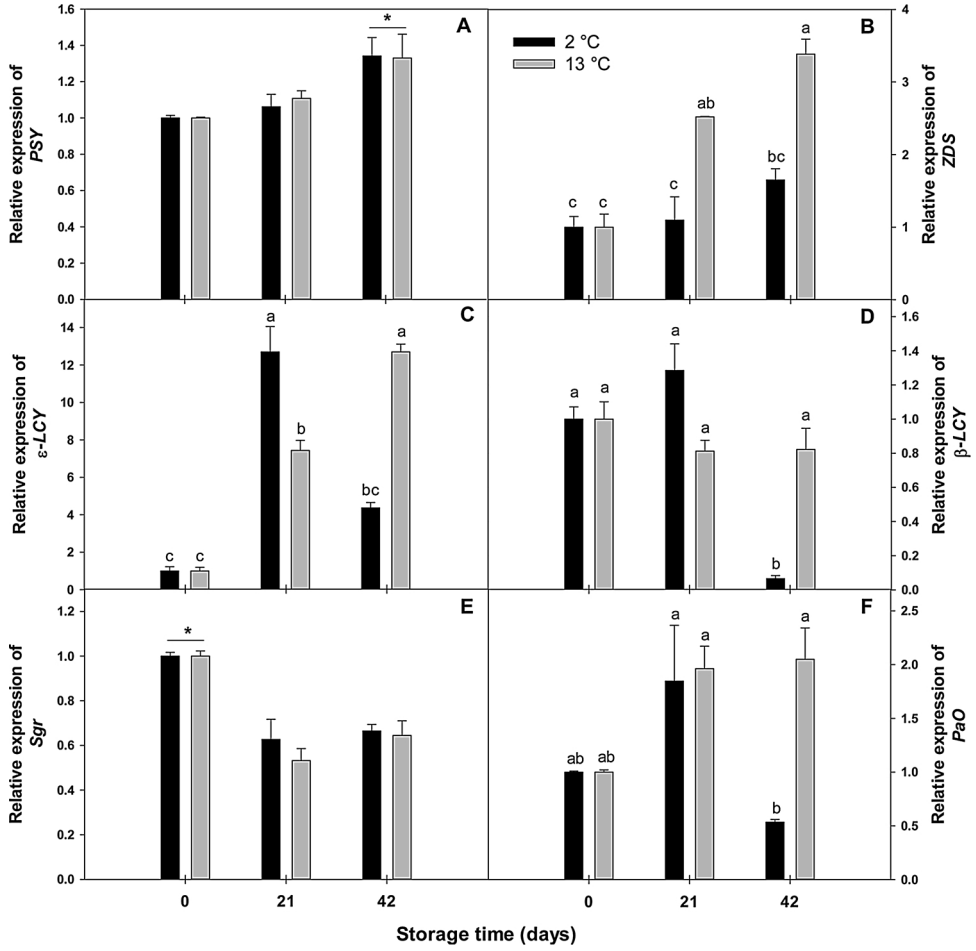

Fig. 3. Relative expression levels of genes associated with the content of pigments in the flavedo of 'Rio Red' grapefruit fruit stored at 2 °C and 13 °C. Carotenoid biosynthesis pathway genes (A to D); Chlorophyll degradation-related genes (E and F). Each value is the mean of three replicate samples ± SE. Mean values with a different letter are significantly different ($P \leq 0.05$). Asterisks indicate significant differences between days of storage ($P \leq 0.05$).

Table 3
ANOVA *P*- and *F*-values for relative expression of genes associated with the content of grapefruit flavedo pigments during postharvest storage at low temperatures.

Factor		*PSY*	*PDS*	*ZDS*	β-*LCY*	ε-*LCY*	*Sgr*	*PaO*
Storage time (A)	*P/F*	0.008‡ /7.98	0.140^{ns} /2.4	< 0.001⁂ /28.56	< 0.001⁂ /17.82	< 0.001⁂ /68.66	< 0.001⁂ /23.61	0.017* /6.63
Storage temperaure (B)	*P/F*	0.875^{ns} /0.03	0.657^{ns} /0.21	< 0.001⁂ /39.91	0.335^{ns} /1.04	0.177^{ns} /2.14	0.461^{ns} /0.59	0.024* /7.31
Interaction (A × B)	*P/F*	0.936^{ns} /0.07	0.465^{ns} /0.83	0.008‡ /10.19	0.001‡ /16.15	< 0.001⁂ /40.51	0.716^{ns} /0.35	0.018* /6.48

^{ns}: non significant ($P > 0.05$).
* Significant at $P < 0.05$.
‡ Significant at $P < 0.01$.
⁂ Significant at $P < 0.001$.

gene expression was not addressed in this work. On the other hand, ε-*LCY* expression levels increased gradually during storage at 13 °C, while at 2 °C levels peaked by day 21 and then decreased to levels comparable with those observed before storage (Fig. 3C). Interestingly, the expression levels of both β-*LCY* and ε-*LCY* genes and the *ZDS* gene are down-regulated after 42 days of storage at 2 °C, which may indicate the content of colored carotenoids β-carotene, α-carotene, and lycopene in the 'Rio Red' grapefruit flavedo are transcriptionally regulated during postharvest storage by temperature and time. These results are consistent with the low CI observed in fruit stored at 2 °C, and may in part explain the observed increase on lycopene accumulation and the enhanced fruit color development during storage at 13 °C.

Previous studies on model citrus fruits reported the effect of storage temperature on the carotenoid biosynthesis regulation. For instance, the accumulation of the *ZDS* transcripts was induced in 'Satsuma' tangerine and 'Navelina' orange by storage at 20 °C and 12 °C, respectively (Matsumoto et al., 2009; Carmona et al., 2012). Particularly, the studies on the postharvest storage of grapefruit fruit have been mainly focused on monitoring phytochemical levels in juice (Chaudhary et al., 2015) and flavedo tissue (Lado et al., 2014). The expression of carotenogenic genes was only addressed during on-tree development and maturation of grapefruit fruit (Alquezar et al., 2013). Therefore, the effect of storage conditions on the regulation of molecular aspects related to the postharvest changes of grapefruit peel color had remained unexplored.

3.4. Expression of genes related to Chl catabolism in response to low temperature storage

The gene expression patterns for *Sgr* and *PaO* are respectively shown in Fig. 3E and F. The Sgr protein is able to extract magnesium from the Chl a ring, while PaO cleaves the remaining porphyrin ring to further produce a colorless compound. *Sgr* transcript levels were significantly higher at the beginning of the experiment and a 30% reduction occurred by days 21 and 42 of storage. Such decrease was not significantly affected by temperature nor storage time-temperature interaction (Table 3). *Sgr* expression decreased during postharvest cold storage in a yellow-fleshed kiwi cultivar, while a more stable expression was observed in green-fleshed cultivars (Gambi et al., 2018). This indicates that *Sgr* expression sensitivity to cold-temperature storage is cultivar-dependent. The ANOVA detected significant differences in the *PaO* gene expression levels for storage time, temperature and for the temperature-storage time interaction. *PaO* transcript levels significantly dropped in fruit stored at 2 °C for 42 days, with respect to levels found in fruit stored at 13 °C, while no differences were noticed by the effect of time and temperature at day 21. Similarly, *PaO* transcript levels were affected by storage temperature in cold-stored kiwi fruit, where lower temperatures reduced its expression (Gambi et al., 2018). These results may explain why, even when Chl levels were gradually decreasing during time of storage, the content of this pigment was higher in fruit stored at 2 °C than at 13 °C (Fig. 1A).

Table 3 resumes whether the evaluated factors influence significantly the expression levels of genes associated to the content of pigments in the peel of grapefruit fruit during its postharvest storage. Briefly, time of storage was the most influencing factor, affecting the expression levels of six out of seven of the evaluated genes. Storage temperature influenced the expression of two genes, while the storage time-temperature interaction significantly affected the expression of four genes. This may be a predictable result for *PaO* and *Sgr*, which are involved in Chl breakdown processes, typical during senescence. However, its influence on the expression of genes of the carotenoid biosynthesis pathway reveals that the postharvest color changes were not only due to degreening, but also to de novo synthesis of carotenoid pigments, previously thought to occur only during fruit maturation.

3.5. Effect of storage temperature on ethylene

Grapefruit stored at 2 °C generated large amounts of ethylene after 21 days compared to fruit stored at 13 °C, with the highest production reached by day 42 (Fig. 4). The increment of ethylene production by cold storage was previously reported for Marsh and Star Ruby grapefruit cultivars (Lado et al., 2014). Ethylene is the product of the expression of the *ACC synthase-ACS1*, *-ACS2*, and *ACC oxidase-ACO* genes, which is known to increase by a variety of stress conditions (Lado et al., 2014). Therefore, the enhanced ethylene production observed at 2 °C may likely have occurred by the chilling stress induced at this storage temperature, which did not influence the lycopene and β-carotene levels, *ZDS* expression and flavedo color. Meanwhile, the low ethylene production in fruit stored at 13 °C suggests either that the observed results may have been mediated mainly by temperature, rather than by sensitivity to ethylene, or that low ethylene levels could be enough to trigger the induction of the *ZDS* gene expression at a storage temperature of 13 °C.

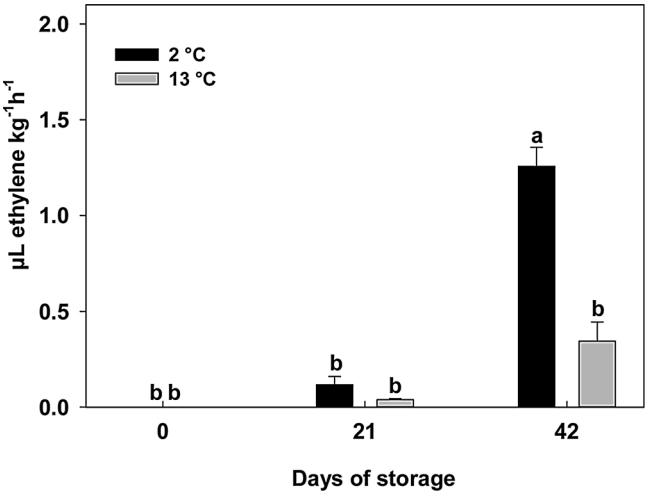

Fig. 4. Ethylene production in 'Rio Red' grapefruit fruit stored at 2 °C and 13 °C; 90–95% RH. Each value is the mean of three replicate samples ± SE. Values with the different letter are significantly different according to Tukey test ($P \leq 0.05$).

4. Conclusion

In summary, the grapefruit quality observed through the color of flavedo during postharvest management is regulated by storage temperature and time. The storage temperature of 13 °C promoted changes in flavedo coloration primarily related to the accumulation of lycopene and degradation of Chl. The increase in the lycopene content and Chl degradation were lower at 2 °C. The lycopene content increase was positively correlated with CI. Storage time and temperature influenced at different extents the expression of genes that regulate both unmasking and de novo synthesis processes of grapefruit peel pigments. Although ethylene levels increased in fruit stored at 2 °C, this hormone did not influence CI. However, the low ethylene production in grapefruit at 13 °C suggests that the observed effects in fruit stored at this temperature may have been mediated mainly by temperature.

Acknowledgements

The authors thank Francisco J. Soto, Cynthia L. Aguilar, and Demetrio Morales for technical support.

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