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ORIGINAL PAPER

A comparative physiological and transcriptional study of carotenoid biosynthesis in white and red grapefruit (*Citrus paradisi* Macf.)

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Abstract Accumulation of lycopene in citrus fruits is an unusual feature restricted to selected mutants. Grapefruit (*Citrus paradisi* Macf.) is the *Citrus* specie with greater number of red-fleshed mutants, but the molecular bases of this alteration are not fully understood. To gain knowledge into the mechanisms implicated in this alteration, we conducted a comparative analysis of carotenoid profile and of the expression of genes related to carotenoid biosynthesis and catabolism in flavedo and pulp of two grapefruit cultivars with marked differences in colouration: the white Marsh and the red Star Ruby. Mature green fruit of Marsh accumulated chloroplastic carotenoids, while mature tissues lacked carotenoids. However, accumulation of downstream products such as abscisic acid (ABA) and expression of its biosynthetic genes, 9-*cis*-epoxycarotenoid dioxygenase (*NCED1* and *NCED2*), increased after the onset of colouration. In contrast, red grapefruit accumulated lycopene, phytoene and phytofluene, while ABA content and *NCED* gene expression were lower than in Marsh, suggesting a blockage in the carotenoid biosynthetic pathway. Expression analysis of three genes of the isoprenoid pathway and nine of the carotenoid biosynthetic pathway revealed virtually no differences in flavedo and pulp between both genotypes, except for the chromoplast-specific lycopene cyclase 2 (*β-LCY2*) which was lower in the pulp of the red grapefruit. The proportion in the expression of the allele with high (*β-LCY2a*) and low (*β-LCY2b*) activity was also similar in the pulp of both genotypes. Therefore, results suggest that reduced expression of β-*LCY2* appears to be responsible of lycopene accumulation in the red Star Ruby grapefruit.

Keywords ABA · Carotenoids · Citrus · Gene expression · Grapefruit · Lycopene

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Introduction

Carotenoids are the main pigments responsible for the attractive colour of the flavedo and pulp of citrus fruits and greatly contribute to their commercial and nutritional value. Fruits of different *Citrus* species display a broad array of colour singularities, from yellow to deep-orange or red, and in many cultivars, the flavedo and the pulp may also exhibit different colouration, envisaging specie- and tissue-specific regulation of the carotenoid content and composition (Alquezar et al. 2008a; Kato 2012).

Regulation of carotenoid accumulation during maturation of orange-coloured fruit has been studied in detail in different citrus varieties, and in most cases, changes in carotenoid content are well correlated with the expression of carotenogenic genes (Kato et al. 2004; Rodrigo et al. 2004; Chen et al. 2010). The flavedo of immature citrus fruit accumulates mainly lutein (ε,β-xanthophyll) and minor proportion of other chloroplastic carotenoids. At the onset of fruit colouration, lutein declines and almost disappears in parallel

with an important accumulation of specific β,β-xanthophylls. The massive increase in total carotenoids during maturation is concomitant with a substantial induction of phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*) and β-carotene hydroxylase (*β-CHX*) gene expression (Kato et al. 2004; Rodrigo et al. 2004; Alós et al. 2006; Alquezar et al. 2008b). The shift from the ε,β-branch to the β,β-branch of the pathway is also coordinated with a downregulation of lycopene ε-cyclase (*ε-LCY*) gene expression (Kato et al. 2004; Rodrigo et al. 2004; Alquezar et al. 2008b). The expression of the chromoplast-specific *β-LCY2* increased also substantially, playing a key role redirecting the flux of carotenes into the β,β-branch and leading the massive accumulation of xanthophylls (Alquezar et al. 2009). In mature oranges, violaxanthin predominates, while in mandarins and their relatives, β-cryptoxanthin is the main carotenoid. This difference in xanthophyll accumulation has been suggested to be related with the relative expression level of *β-CHX* gene (Kato et al. 2004). In the pulp of oranges and mandarins, total carotenoid content is usually lower than in the flavedo, in accordance with a reduced expression of most carotenoid biosynthetic genes (Kato et al. 2004; Alquezar et al. 2009). Recently, it has been suggested that alterations in the expression of genes related to the MEP pathway (Alquezar et al. 2008b) or the antioxidant enzymatic system (Yu et al. 2012) could also influence carotenoid content and composition in lycopene-accumulating orange mutants.

Fanciullino et al. (2006) analysed carotenoid content and composition in 25 citrus genotypes and pointed out that the variability observed was more interspecific than intraspecific. Based on carotenoid diversity, genotypes were classified in three clusters that matched previous phylogenetic classifications. Nowadays, it is considered that the three pure *Citrus* species are citron (*Citrus medica* L.), mandarin (*Citrus reticulata* Blanco) and pummelo (*Citrus grandis* (L.) Osb.) (Nicolosi et al. 2000; Gmitter et al. 2012; Garcia-Lor et al. 2013). Each of these taxa is characterized by a 'typical' colour, e.g. mandarin and its relatives are orange, pummelo flavedo and pulp are yellow to white, while citron are of yellow colour. Cultivated genotypes are likely derived from hybridization between these true species, high frequency of spontaneous bud mutations and the long history of cultivation and worldwide dispersion (Gmitter 1995).

Analysis of the expression of carotenoid biosynthetic genes in yellow-coloured *Citrus* fruits is still limited. The flavedo of Lisbon lemon accumulates large amount of the colourless phytoene and phytofluene, but the pulp is almost lacking of carotenoids (Kato et al. 2004). This particular complement of carotenoids appears to be related to a reduced expression of the *PDS* gene in the flavedo and to the whole set of carotenogenic genes required to produce xanthophylls (Kato et al. 2004). Marsh seedless is the most cultivated white grapefruit in the world and the primary source of diversification from which most of the pigmented (pink- and red-coloured) grapefruit varieties have been derived and selected (Corazza Nunes et al. 2002). Marsh grapefruit accumulated only scarce amounts of phytoene and phytofluene during maturation in both flavedo and pulp (Yokoyama and White 1967; Banet et al. 1981; Xu et al. 2006a; Matsumoto et al. 2007), a defect that has been for many years attributed to a deficient phytofluene desaturation (Yokoyama and White 1967; Romojaro et al. 1979; Banet et al. 1981; Gross 1987). However, the molecular basis of this phenotypic peculiarity is not yet understood. Early investigations demonstrated that coloured grapefruits contained lycopene and β-carotene in either flavedo and pulp (Matlack 1935; Khan and Mackinney 1953), and a correlation between lycopene content and the intensity of pulp colouration was established decades ago (Matlack 1935; Ting and Deszyck 1958). Lycopene accumulation is an unusual feature in most citrus fruits since it has been only identified in *Citrus paradisi* and *C. grandis* and three mutants in *Citrus sinensis* (Monselise and Halevy 1961; Saunt 2000; Xu et al. 2006a; Liu et al. 2007; Alquezar et al. 2008b). In the flavedo and pulp of red-coloured grapefruits, the most abundant pigments are carotenes, mainly phytoene, phytofluene, lycopene and β-carotene (Khan and Mackinney 1953; Curl and Bailey 1957; Gross 1987; Fanciullino et al. 2006; Xu et al. 2006a; Matsumoto et al. 2007; Alquezar et al. 2009). It is noteworthy that in these red phenotypes, total carotenoid content is very similar in the flavedo and pulp, an unusual feature in citrus and, in general, in carotenogenic fruits (Gross 1987). It has been hypothesized that lycopene accumulation in red grapefruits could be related to lycopene sequestration structures (Xu et al. 2006a) or to a blockage at the level of β-carotene (Fanciullino et al. 2006). In the red grapefruit Star Ruby, accumulation of lycopene and β-carotene during maturation was associated with a reduced expression of *β-LCY2* and *β-CHX* genes in comparison with Navel oranges. Moreover, pulp of red grapefruit expressed predominately a *β-LCY2* allele with reduced activity (Alquezar et al. 2009). It has been recently reported that the expression of *β-LCY2* gene is at least 71 % lower in the red-fleshed Flame grapefruit than in the white Marsh grapefruit (Mendes et al. 2011). In addition, a higher level of *PSY* mRNA was detected in Flame grapefruit than in the white Duncan grapefruit (Costa et al. 2011).

The objective of the present study has been to understand whether differences in colouration and in carotenoid content and composition in the flavedo and pulp of white (Marsh) and red (Star Ruby) grapefruit are related to differential expression of carotenoid biosynthetic genes. We report a global view of the changes in carotenoid content and composition during fruit maturation and the expression of biosynthetic genes of the whole metabolic pathway, including those of the plastidic MEP pathway (*DXS*, *HDS* and *HDR*), and of carotenes and xanthophylls biosynthesis (*PSY*, *PDS*, *ZDS*, *ε-LCY*, *ε-CHX*, *β-LCY1*, *β-LCY2*, *β-CHX* and *ZEP*).

Our working hypothesis envisages a partial blockage of the carotenoid biosynthetic pathway in the lycopene-accumulating grapefruit that may reduce the flow to downstream metabolites of the pathway. Therefore, to ascertain this hypothesis, the concentration of abscisic acid (ABA) and the expression of two key genes of its biosynthesis (9-*cis*-epoxycarotenoid dioxygenase, *NCED1* and *NCED2*) in fruit of both grapefruit during maturation were determined as catabolism of carotenoids has been also postulated to play an important role in regulation of carotenoid biosynthesis and accumulation in citrus fruits (Kato et al. 2006).

Material and methods

Plant material and fruit colouration

Fruits of both white Marsh and the red-fleshed Star Ruby grapefruits (*C. paradisi* Macf.) at eight developmental stages (Fig. 1), from immature-green to full-coloured, were harvested at random from outer canopy of adult trees grafted on Citrange carrizo (*C. sinensis*×*Poncirus trifoliata*) rootstocks cultivated at The Citrus Germplasm Bank (Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain) during two consecutive seasons. Fruits were immediately delivered to the laboratory, and fruit colour was measured using a Minolta CR-330 chromameter on three locations around the equatorial plane of the fruit. Hunter parameters *a* (negative to positive correspond from green to red, respectively) and *b* (negative to positive, from blue to yellow, respectively) were determined, and colour was expressed as the *a*/*b* Hunter ratio, a classical relationship for colour measurement in citrus fruits (Stewart and Wheaton 1972). Negative *a*/*b* values represent green colour, 0 is the breaker point and positive values indicate yellow to orange colouration. Fruit colour was registered using 30 replicate samples for each developmental stage and genotype. Flavedo (the outer coloured part of the fruit peel) and pulp were separated with a scalpel, immediately frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis. All experiments were conducted at least twice with samples from two independent seasons. Results presented are representative data from two seasons.

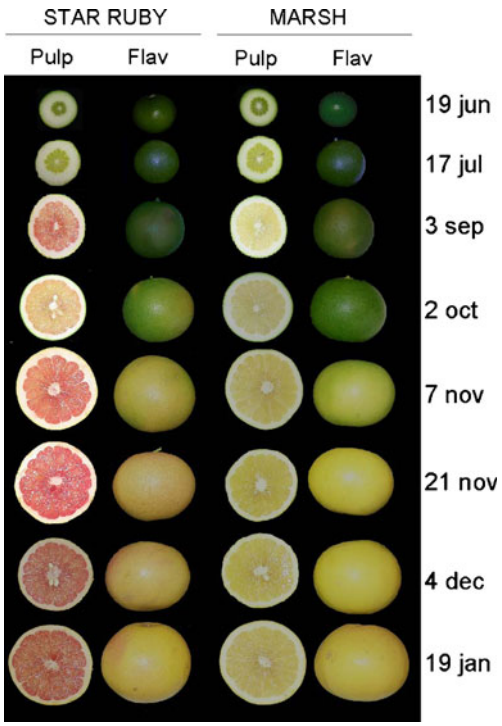

Fig. 1 Internal (pulp) and external (flavedo) appearance of Star Ruby and Marsh fruits during development and maturation. Sampling data are indicated on the *right*

Chlorophyll and total carotenoid extraction and quantification

Freeze ground material of flavedo or pulp was used for total chlorophyll and carotenoid extraction, following the protocol described by Rodrigo et al. (2003). Briefly, freeze ground material (500 mg) of flavedo or pulp was extracted with a mixture of methanol and 50 mM Tris–HCl buffer (pH 7.5) containing 1 M NaCl and partitioned against chloroform until plant material was uncoloured. Pooled organic phases were dried under vacuum and saponified overnight using a KOH methanolic solution. The carotenoids were subsequently re-extracted with diethyl ether. An aliquot of the ethereal extract was used for quantification of total carotenoid content. Absorption spectra of saponified extracts were recorded with a Diode array spectrophotometer (model 8452A Hewlett Packard, Germany). The maximum absorbance peaks were registered, and total carotenoid content was calculated by measuring the absorbance at 450 nm according to Davies (1976), using the extinction coefficient of β-carotene, $E^{1\%}$=2,500. The samples were dried under N_2 and kept at −20 °C until high-performance liquid chromatography (HPLC) analysis. Each sample was extracted at least three times. All operations were carried out on ice under dim light to prevent photodegradation, isomerisations and structural changes of carotenoids.

HPLC analysis of carotenoids

Carotenoid composition of each sample was analysed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a model 996 photodiode array detector, and Empower software (Waters, Barcelona, Spain), as described previously (Alquezar et al. 2008b). Carotenoids were identified by their retention time, absorption and fine spectra. For each elution, a Maxplot chromatogram was obtained, which plots each carotenoid peak at its corresponding maximum absorbance wavelength. The

carotenoid peaks were integrated at their individual maxima wavelength, and their content was calculated using calibration curves of zeaxanthin (Sigma) for zeaxanthin, β-carotene (Sigma) for α-carotene and β-carotene, lycopene (Sigma) for lycopene, β-cryptoxanthin (Extrasynthese) for α-cryptoxanthin and β-cryptoxanthin, lutein (Sigma) for lutein, violaxanthin and neoxanthin isomers and β-apo-8′-carotenal (a gift from Hoffman-LaRoche) for β-citraurin. Phytoene and phytofluene were previously purified as is described in Pascual et al. (1993) by thin-layer chromatography from carotenoid extracts of Pinalate orange fruit, a mutant which accumulates substantial amounts of these carotenes (Rodrigo et al. 2003).

ABA analysis

Quantification of ABA in flavedo and pulp tissue was performed by indirect enzyme-linked immunosorbent assay as reported previously (Zacarias et al. 1995; Lafuente et al. 1997).

Total RNA isolation and Northern blot hybridization

Plant material used for total RNA isolation was the same as that used for chlorophyll and carotenoid analysis. Total RNA extraction from flavedo and pulp, and Northern blotting hybridization were performed as described previously (Rodrigo et al. 2004). Probes were derived from cDNA clones of the carotenoid biosynthetic genes *PSY*, *PDS*, *ZDS*, *β-LCY1*, *β-LCY*, *β-CHX*, *ZEP* (Rodrigo et al. 2004) and *β-LCY2* and *β-CHX* (Alquezar et al. 2009), MEP pathway genes *DXS*, *HDS* and *HDR* (Alquezar et al. 2008b), *NCED*s genes (Rodrigo et al. 2006) and 26rDNA (Ballester et al. 2006) from *C. sinensis*. All probes were labeled with [α-32P]dATP by linear PCR amplification using the Strip-EZ PCR Kit (Ambion, Huntingdon, UK) following the instructions of the manufacturer. An equivalent number of counts (10^6 cpm ml^{-1}) were used for each hybridization.

Northern blots were exposed to Phosphorscreens and the images read on a FLA-3000 laser scanner (Fujifilm, Tokyo, Japan). In order to determine relative gene expression, signal in each band was determined using ImageGauge 4.0 (Fujifilm) software. Filters were stripped off following the instructions in the Strip-EZ PCR kit and re-hybridized several times. Finally, filters were hybridized to the 26S rDNA *C. sinensis* probe to normalize the hybridization of each gene by calculating the ratio between the hybridization signal of each mRNA and that obtained using the 26S rDNA *C. sinensis* probe. For each gene, a value of 100 was assigned to the normalized signal of Star Ruby flavedo at full-coloured stage and expression level of the remained samples referred to it.

qRT-PCR analysis of *β-LCY2* gene

To analyse the expression on the *β-LCY2* gene in the pulp of Marsh and Star Ruby grapefruit, total RNA was treated with DNase (Ambion, Huntingdon, UK) and accurately quantified. Quantitative real-time was performed as described previously by Alquezar et al. (2009) for the same gene. To transform fluorescence intensity measurements into relative mRNA levels, a tenfold dilution series of a RNA sample was used as a standard curve. Values were the mean of at least three independent analyses.

To analyse the relative expression of the two alleles, *β-LCY2a* and *β-LCY2b*, of the *β-LCY2* gene, specific primers were designed in the region of high sequence variability between both alleles. Primer pairs for *β-LCY2a* allele were; MJ426 (sense) 5′-GAGCAAGTCTCATCGCGTCATAGTG-3′ and MJ427 (antisense) 5′-ACTTTAGCCTTATGAAACTTAACTCCATTTG-3′ and for *β-LCY2b* allele; MJ430 (sense) 5′-GCAAGTCTCATCGCGTCATGGTA-3′ and MJ431 (antisense) 5′-ACTTTAGCCTTATGAAACCTAACGCCATTTA-3′. The cycling protocol for both alleles consisted of 10 min at 95 ° C for pre-incubation, then 40 cycles of 10 s at 95 ° C for denaturation, 10 sat 59 °C for annealing and 10 s at 72 °C for extension. Fluorescent intensity data were acquired during the extension time. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps. For expression measurements, the absolute quantification analysis from the LightCycler 480 Software (Roche, release 1.5.0, version 1.5.0.39) was used, and the expression level relative to the values of a reference sample was calculated using the Relative Expression Software Tool (Pfaffl et al. 2002). Normalization was performed using the expression levels of the actin gene (Romero et al. 2012). Values were the mean of at least three independent analyses.

Data analysis

When appropriate, one-way analysis of variance was performed using the Statgraphics v.5.1 Software (Manugistics, Inc.). A Fisher's Protected LSD test ($P \leq 0.05$) was employed to determine significant differences.

Results

Evolution of fruit colour, chlorophyll and carotenoid content in flavedo and pulp of Marsh and Star Ruby grapefruit during development and maturation

Marsh and Star Ruby grapefruit were harvested at eight different developmental/maturation stages, from middle

June to middle January (Figs. 1 and 2). Under Mediterranean climatic conditions, flavedo of both genotypes remained green until beginning of October and then started to degreen to attain the final colour latter December–early January (Figs. 1 and 2a). Flavedo of Marsh grapefruit was of bright-yellow colouration, while that of Star Ruby developed pink-reddish sectors over a yellow background of the surface. Colour of the pulp remained nearly unchanged in Marsh grapefruit during the whole development and maturation but in Star Ruby grapefruit developed a typical red colour and thus resulting in higher *a*/*b* Hunter ratio (Figs. 1 and 2b). In immature green (IG) fruits, the concentration of chlorophyll (Chl) in the flavedo of Marsh almost doubled that of Star Ruby, but its degradation started earlier, and by early September, the decline in Chl content followed a similar evolution in the flavedo of both genotypes (Fig. 2c). Chl content in the pulp was much lower than in the flavedo, and after September, its concentration was almost negligible (Fig. 2d)

During development and maturation, total carotenoid content (calculated as micrograms of β-carotene equivalents) diminished in the flavedo of both genotypes in a similar fashion to values between 5 and 8 μg/g FW in mature fruits (Fig. 2e). In the pulp, evolution of carotenoid content was markedly different between each grapefruit (Fig. 2f). In Marsh grapefruit, carotenoid concentration was very low and nearly constant through the whole process. In the pulp of the red Star Ruby, two peaks of

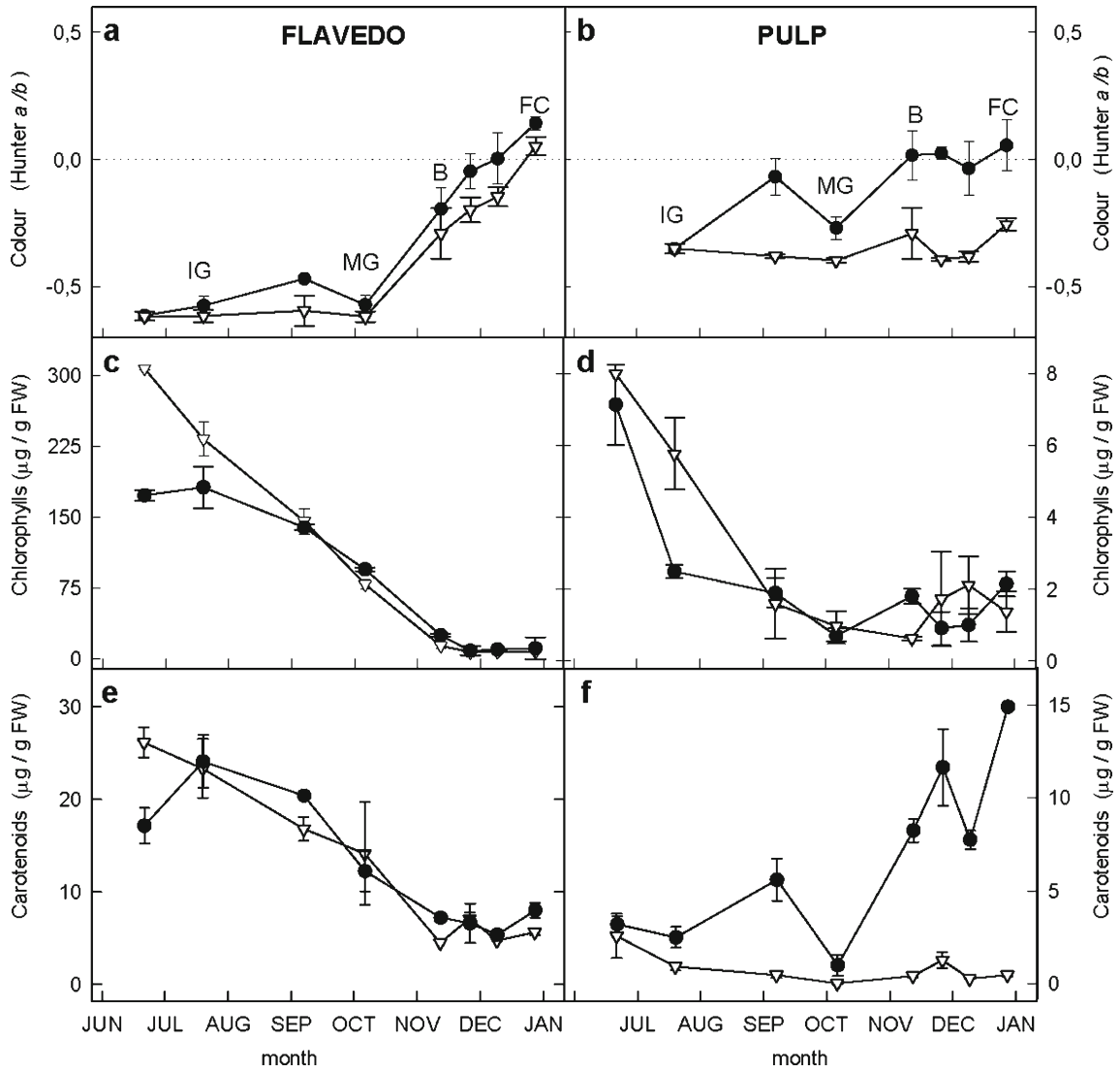

Fig. 2 Evolution of flavedo and pulp colour (**a**, **b**) and chlorophyll (**c**, **d**) and carotenoid contents (**e**, **f**) in the flavedo (*left panels*) and in pulp (*right panels*) during development and maturation of Star Ruby (*black circle*) and Marsh (*white triangle*) fruits (*C. paradisi* Macf.). The *dotted line* indicates the colour index at the colour break. The harvest time of the fruit at *IG* (Immature-green), *MG* (Mature-green), *B* (Breaker) and *FC* (Full-colour) stage is indicated. Fruit colour is expressed as the *a*/*b* Hunter ratio, and data are the means±SD of 30 replicates. Data of Chl and total carotenoid content are the means±SD of at least three independent measurements

carotenoid content were found, one at the end of the cell enlargement phase (September) and a second after colour-break, to reach in full mature fruit a concentration of about 15 μg/g FW, which was almost 25 times higher than that of the pulp of Marsh and almost double than of its flavedo (Fig. 2f)

Carotenoid composition in flavedo and pulp during development and maturation of Marsh and Star Ruby grapefruit

Changes in the composition of carotenoids in flavedo and pulp of Marsh and Star Ruby grapefruit at four developmental and ripening stages (immature-green, IG; mature-green, MG; breaker, B; and full-coloured, FC, Fig. 2) were analysed by HPLC-PDA. Nine major carotenoids were quantified, accounting for more than 98 % of total carotenoids in all samples analysed. In flavedo of green Marsh and Star Ruby fruits (IG and MG stages) chloroplastic carotenoids dominated, being lutein the most abundant in both genotypes (Fig. 3). Linear carotenes (mainly phytoene) also accumulated, accounting for more than 30 % of total carotenoids. During maturation of both grapefruits, the concentration of chloroplastic carotenoids decreased. The flavedo of mature fruit of Marsh only contained minute amounts of phytoene, violaxanthin and zeaxanthin. In the flavedo of Star Ruby, by contrast, the content of linear carotenes, mainly phytoene, but also phytofluene and lycopene, increased substantially during maturation, accounting for

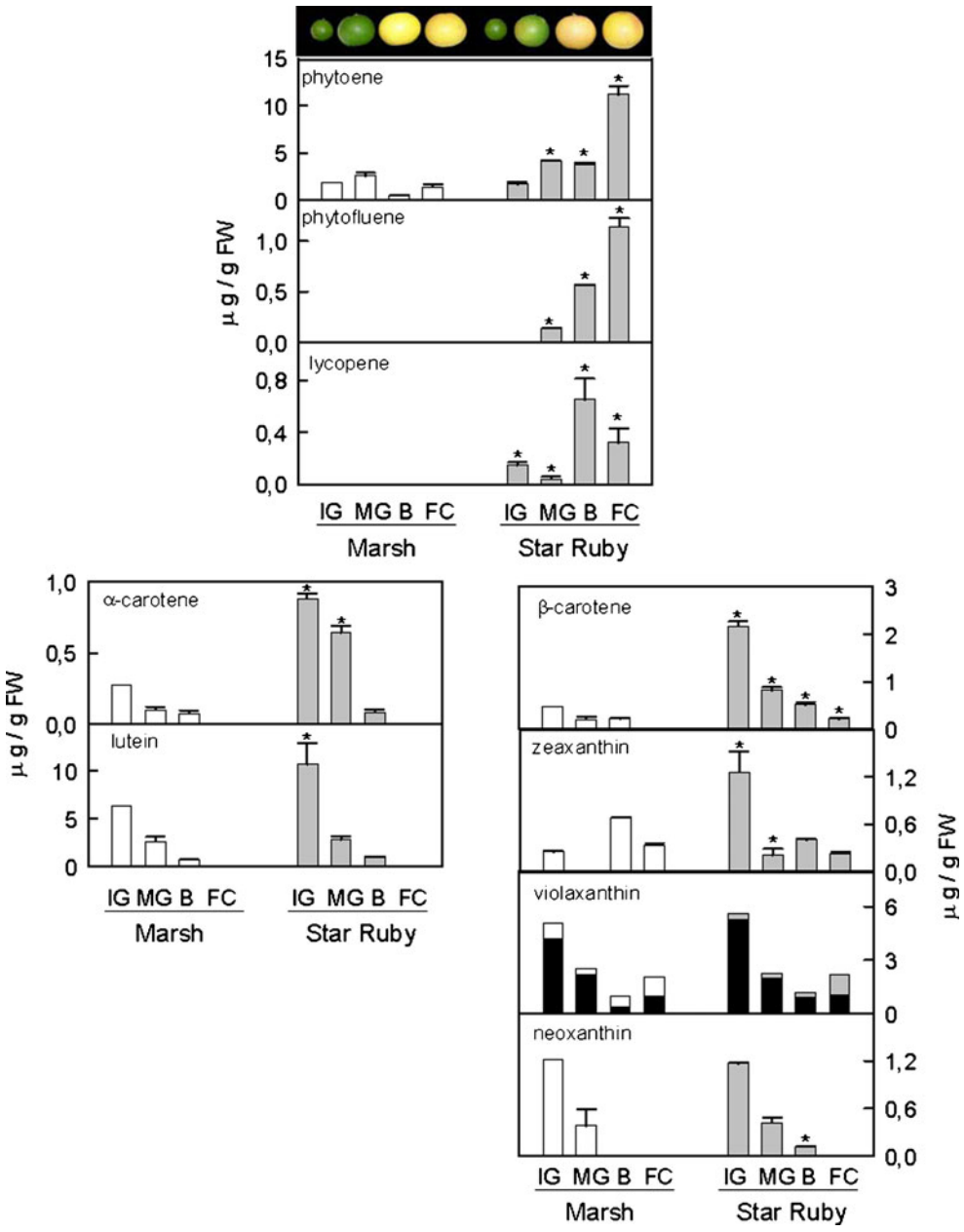

Fig. 3 External appearance and carotenoid composition in the flavedo of Star Ruby and Marsh fruits (*C. paradisi* Macf.) at *IG* (Immature-green), *MG* (Mature-green), *B* (Breaker) and *FC* (Full-colour) stages. The plots were arranged following the carotenoid biosynthetic sequence in the pathway. When *E*- and *Z*-isomers of a particular carotenoid are identified, the *E*-isomer is represented in *black colour*. The data are means±SD of at least three independent measurements. Significant differences ($P \leq 0.05$) in individual carotenoids between Marsh and Star Ruby flavedo samples for the same maturity stage are indicated by an *asterisk*

approximately 84 % of the total carotenoids (Fig. 3). Small concentration of violaxanthin and, to a lesser extent, β-carotene and zeaxanthin was also detected (Fig. 3).

In the pulp of green fruit (IG and MG) of both genotypes, almost negligible amounts of carotenoids were detected (Fig. 4). During maturation, marked differences were observed between both grapefruits. In the pulp of Marsh grapefruit, carotenoid content decreased to a very low level (below 1 μg/g FW), being only detectable phytoene and violaxanthin in trace amounts. Contrary, carotenoid content in the pulp of the red grapefruit increased during maturation. Most abundant carotenoids were phytoene and lycopene, which accounted for between 25–55 and 42–53 % of total carotenoids, respectively. It is remarkable that after the breaker stage no xanthophylls were detected in the pulp of Star Ruby (Fig. 4). As expected, the red pigment lycopene never was detected in flavedo and pulp extracts of Marsh grapefruit.

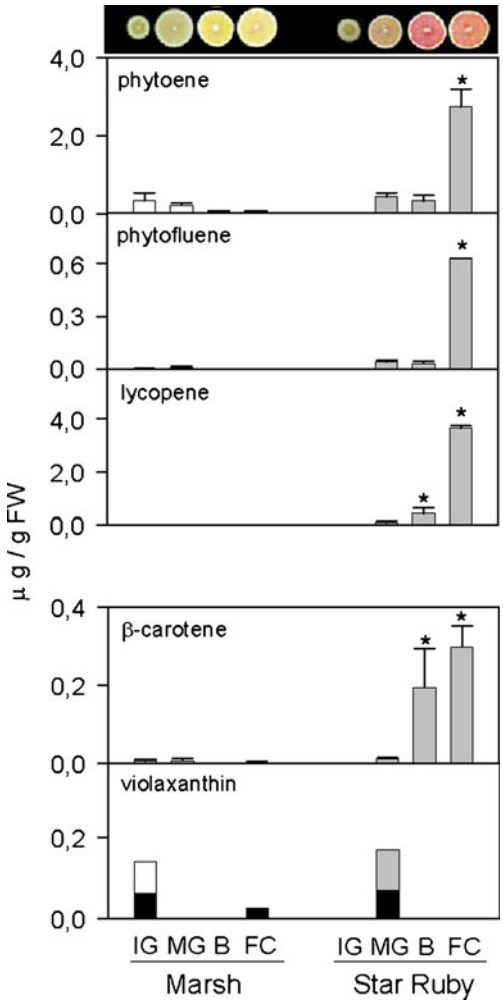

Fig. 4 Internal appearance and carotenoid composition in the pulp of Star Ruby and Marsh fruits (*C. paradisi* Macf.) at *IG* (Immature-green), *MG* (Mature-green), *B* (Breaker) and *FC* (Full-colour) stages. The plots were arranged following the carotenoid biosynthetic sequence in the pathway. When *E*- and *Z*-isomers of a particular carotenoid are identified, the *E*-isomer is represented in *black colour*. The data are means±SD of at least three independent measurements. Significant differences ($P \leq 0.05$) in individual carotenoids between Marsh and Star Ruby pulp samples for the same maturity stage are indicated by an *asterisk*

Expression of isoprenoid and carotenoid biosynthetic genes in flavedo and pulp during development and maturation of Marsh and Star Ruby grapefruit

To examine regulation of carotenoid biosynthesis in white and red grapefruit and how differences in the carotenoid complement may be linked to differential changes in gene expression, accumulation of transcripts corresponding to three genes of the MEP pathway and nine genes of the carotenoid biosynthetic pathway was analysed in the flavedo and pulp of Marsh and Star Ruby fruits at four developmental stages (Figs. 5 and 6). In both grapefruits, expression profile of *DXS* and *HDR* was clearly different between flavedo and pulp. In the flavedo, transcripts of *DXS* and *HDR* were high in green fruits to decline during maturation. By contrast, transcripts of these genes in the pulp increased at the B stage and remained at relative high level in coloured fruits. The pattern of expression of *HDS* gene was similar in both tissues and genotypes and increased during maturation. Interestingly, in the respective tissue of FC fruit, expression of *HDS* gene was always higher in Marsh than in Star Ruby (Fig. 5).

In flavedo and pulp of both grapefruits, expression of early carotenogenic genes (*PSY*, *PDS*, *ZDS*) increased progressively from green fruit to reach a maximum at the breaker stage and remained fairly constant thereafter (Fig. 6). It is interesting to note that, after initiation of ripening, accumulation of *ZDS* transcript was higher in Marsh than in Star Ruby tissues. Regarding the genes of the α,β- and β,β-branch, except for *β-LCY2*, similar expression profiles were also detected in Marsh and Star Ruby grapefruits. In flavedo and pulp, expression of *ε-LCY* and *ε-CHX* genes, involved in lutein biosynthesis, declined during maturation. *β-LCY1* mRNAs increased in the flavedo of both grapefruits, showing a transitory decrease in Marsh at the MG stage, while in the pulp remained low and relatively constant. The expression of *β-CHX* and *ZEP* genes increased during maturation, but to a higher extent in the flavedo than in the pulp. *β-LCY2* gene experienced an important stimulation in the flavedo of both genotypes. In the pulp, its expression showed a maximum at the breaker stage and, interestingly, it was higher in the white Marsh than in the red-fleshed Star Ruby (Fig. 6).

Since expression of *β-LCY2* gene has been postulated to play a relevant role in the synthesis of lycopene in *Citrus* (Alquezar et al. 2009; Mendes et al. 2011), its pattern of expression in the pulp of both grapefruits was further analysed by qRT-PCR analysis. Results confirmed those of Northern

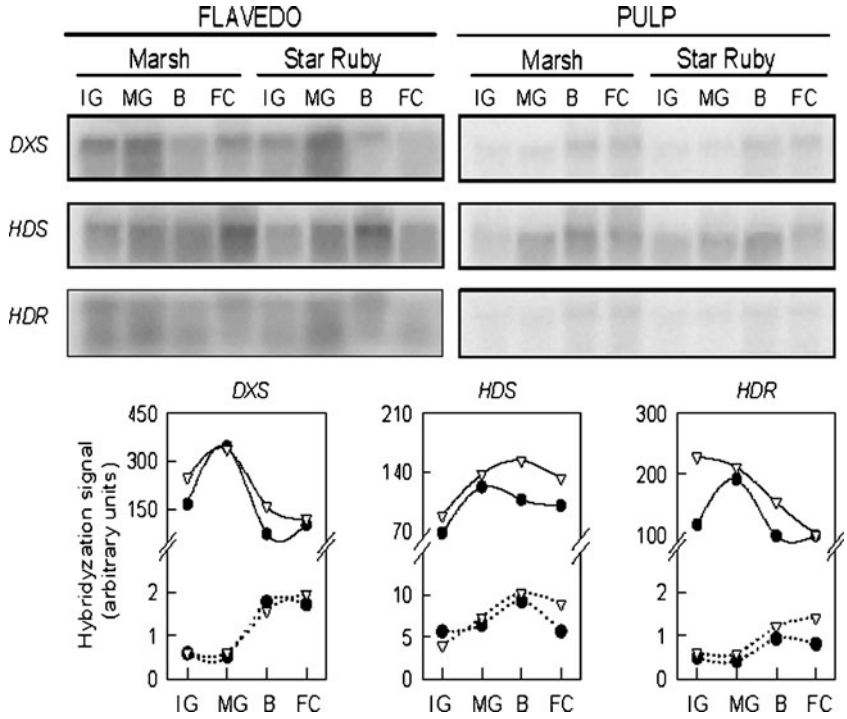

Fig. 5 Accumulation of mRNAs corresponding to *DXS*, *HDS* and *HDR* genes from MEP pathway in the flavedo (*solid line*) and the pulp (*dashed line*) of Star Ruby (*black circle*) and Marsh (*white triangle*) fruits (*C. paradisi* Macf.) at the *IG* (Immature-green), *MG* (Mature-green), *B* (Breaker) and *FC* (Full-colour) stages. All transcripts values for individual genes were normalized with respect to the corresponding value of the 26s rRNA signal. Normalized values of mRNAs accumulation in arbitrary units are represented at the *left*, using the FC flavedo of Star Ruby as a reference (100). Expression data are representative of two independent experiments

blot, showing that after the IG stage accumulation of *β-LCY2* mRNA was substantially higher (between 1.5 to 2.5 times) in the pulp of Marsh grapefruit than in that of Star Ruby (Fig. 7). Moreover, the relative expression of the two alleles of *β-LCY2* gene in the pulp of both genotypes was also examined since the expression of the allele with high in vitro lycopene cyclase activity (*β-LCY2a*) has been shown to be substantially higher in Navel oranges than in lycopene-accumulating grapefruit

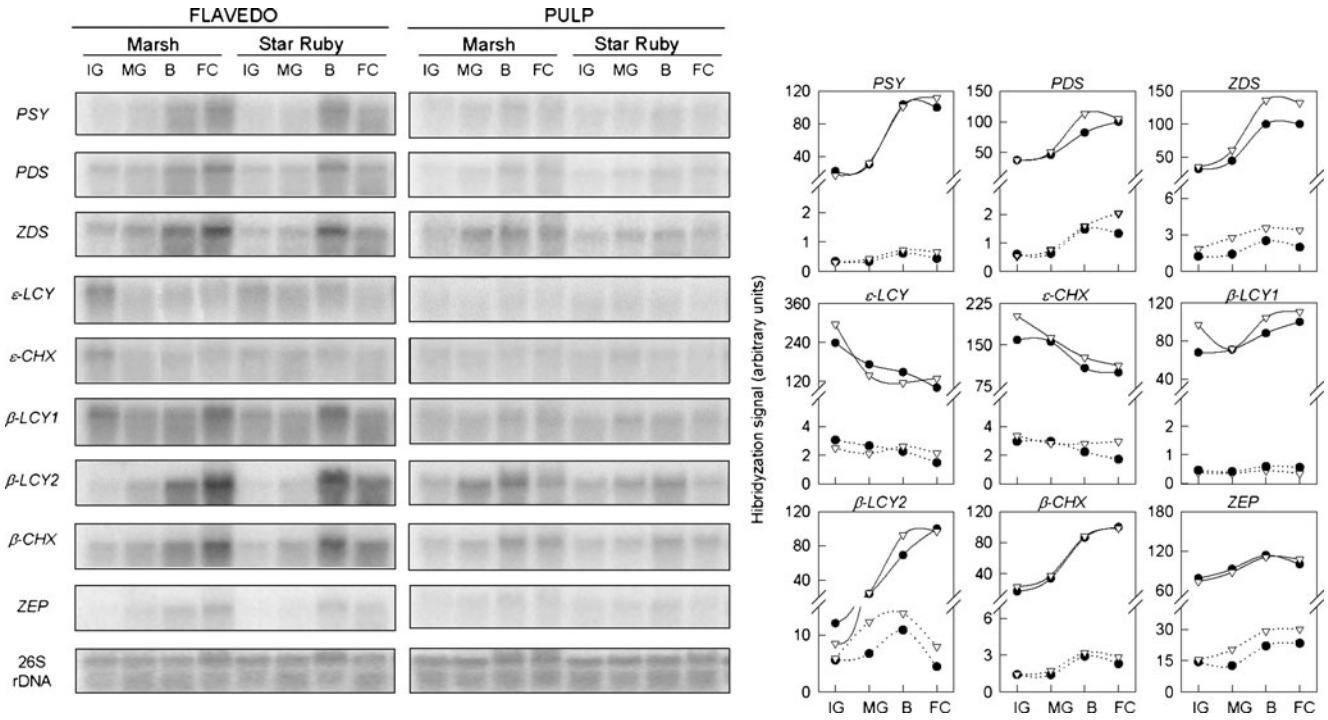

Fig. 6 Accumulation of mRNAs corresponding to *PSY*, *PDS*, *ZDS*, *ε-LCY*, *ε-CHX*, *β-LCY1*, *β-LCY2*, *β-CHX* and *ZEP* genes in the flavedo (*solid line*) and the pulp (*dashed line*) of Star Ruby (*black circle*) and Marsh (*white triangle*) fruits (*C. paradisi* Macf.) at the *IG* (Immature-green), *MG* (Mature-green), *B* (Breaker) and *FC* (Full-colour) stages. All transcripts values for individual genes were normalized with respect to the corresponding value of the 26s rRNA signal. Normalized values of mRNAs accumulation in arbitrary units are represented at the *left*, using the FC flavedo of Star Ruby as a reference (100). Expression data are representative of two independent experiments

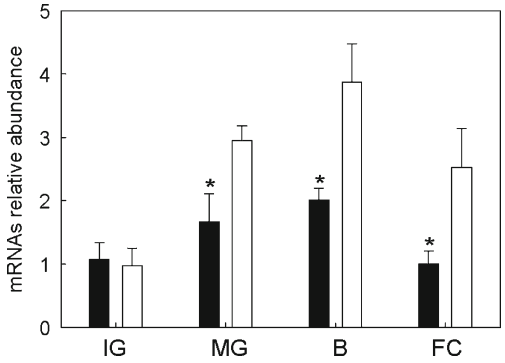

Fig. 7 Quantitative RT-PCR analysis of the expression of *β-LCY2* gene in the pulp of Star Ruby (*black square*) and Marsh (*white square*) fruits (*C. paradisi* Macf.) at the *IG* (Immature green), *MG* (Mature green), *B* (Breaker) and *FC* (Full-colour) stages. The levels of expression were normalized to the amount of RNA, and the value of Star Ruby pulp at the IG stage was set to 1. The data are means±SD of three experimental replicates. Significant differences ($P \leq 0.05$) in the expression level between Marsh and Star Ruby pulp samples for the same maturity stage are indicated by an *asterisk*

(Alquezar et al. 2009). Using allele-specific primers, it was detected that the proportion in the expression of the alleles with high/low activity was very similar in the pulp of the white and red grapefruit at different ripening stages. Thus, at the breaker stage, *β-LCY2* transcripts corresponding to *β-LCY2a* allele were 54.5±7.1 and 52.2±3.7 % for Marsh and Star Ruby, respectively. In coloured fruit, the proportion of *β-LCY2a* transcripts was also similar in both genotypes (48.4±3.6 % in Marsh and 50.9±4.2 % in Star Ruby).

Analysis of ABA content and expression of ABA biosynthetic genes in flavedo and pulp during development and maturation of Marsh and Star Ruby grapefruit

In flavedo of green fruits of both genotypes, ABA content was very low and increased progressively after the colour break. ABA content in the flavedo of full mature fruit of the white Marsh grapefruit was almost double than that of the red Star Ruby (Fig. 8). In pulp, ABA content was also significantly higher in the white than in the red grapefruit throughout the whole period (Fig. 8).

Expression of the two *NCED* (9-*cis*-epoxycarotenoid dioxygenase) genes involved in ABA biosynthesis in *Citrus* fruits was determined in flavedo and pulp of both grapefruits. Expression of *NCED1* and *NCED2* genes in the flavedo increased during maturation of both grapefruits reaching a maximum at the FC stage. The stimulation of *NCED1* in the flavedo of Marsh preceded that of Star Ruby and remained always at higher level. Regarding the pulp, different expression profiles were detected for each *NCED* gene. The level of *NCED1* mRNA increased after MG and, as in the flavedo, it was higher in Marsh than in Star Ruby. Accumulation of the *NCED2* mRNA decreased steadily from the IG to the B stage and sharply at the FC stage, but it was also always higher in the pulp of Marsh than in Star Ruby grapefruit (Fig. 8). Results reinforce the notion that a lower ABA concentration in the pulp of red grapefruit is associated with a reduced expression of both *NCED1* and *NCED2* genes.

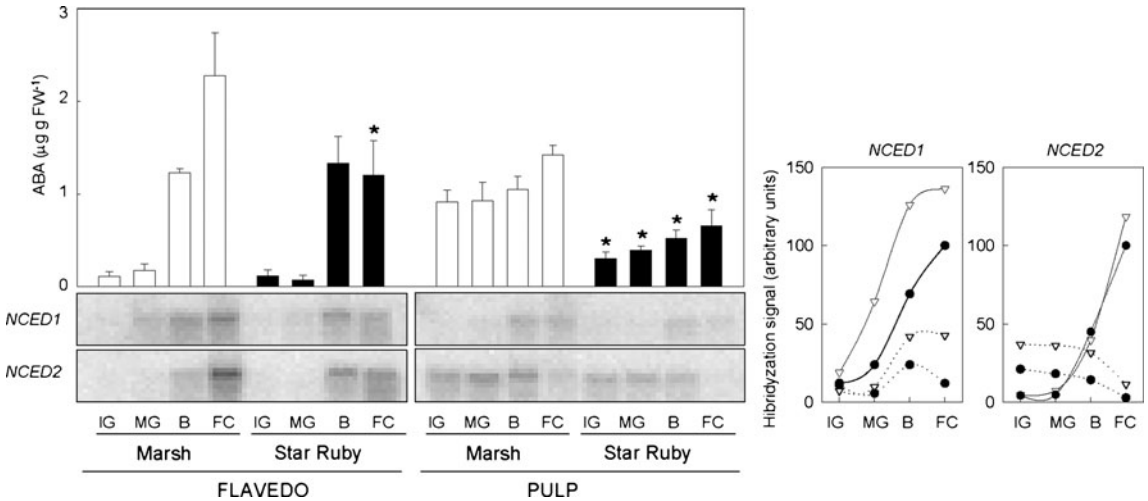

Fig. 8 ABA content and accumulation of mRNAs from *NCED1* and *NCED2* genes in the flavedo (*solid line*) and the pulp (*dashed line*) of Star Ruby (*black circle*) and Marsh (*white triangle*) fruits (*C. paradisi* Macf.) at the *IG* (Immature-green), *MG* (Mature-green), *B* (Breaker) and *FC* (Full-colour) stages. All transcripts values for individual genes were normalized with respect to the corresponding value of the 26s rRNA signal. Normalized values of mRNAs accumulation in arbitrary units are represented at the *left*, using the FC flavedo of Star Ruby as a reference (100). ABA content is the mean±SD of three replicates, and expression data are representative of two independent experiments. Significant differences ($P \leq 0.05$) in ABA content between Marsh and Star Ruby samples for the same maturity stage are indicated by an *asterisk*

Discussion

Accumulation of lycopene is an unusual feature in citrus fruits, being grapefruit (*C. paradisi*) and pummelos (*Citrus maxima*) among the *Citrus* species in which greater number of red mutants has been described (Gmitter 1995). In other *Citrus* species, such as sweet orange (*C. sinensis*), only few red-fleshed mutants have been up-to-date identified, such as 'Hong Anliu' in China (Liu et al. 2007) or 'Cara Cara' in Venezuela (Lee 2001). Analysis of the carotenoid content and complement in the pulp of such spontaneous bud-orange mutants during fruit maturation revealed that the basis of the alteration leading to lycopene accumulation is different between each other and also compared with red-coloured grapefruits (Xu et al. 2006a, b; Liu et al. 2007; Alquezar et al. 2008b; Yu et al. 2012). Understanding the basis of lycopene accumulation in *Citrus* would be of paramount importance since the relevance of this carotene for human nutrition and health is well documented (Rao and Rao 2007) and also considering the high consumption of citrus worldwide, as both fresh fruit and juice. To unveil carotenoid biosynthetic genes underlying lycopene accumulation in red grapefruit, in this study we conducted a comparative analysis of carotenoid content and composition, and also of the expression of genes of the biosynthetic pathway in flavedo and pulp of a white (Marsh) and a red-coloured (Star Ruby) grapefruit during fruit ripening. Moreover, we also determined ABA content and expression of key biosynthetic genes in order to ascertain if the potential alteration in the biosynthetic pathway of carotenoids may have caused a blockage which reduces the flow to downstream products of the pathway.

Star Ruby was selected as red-pigmented grapefruit because its flesh is recognized by having one of the highest lycopene content compared with other red-pigmented citrus cultivars (Xu et al. 2006a, b). By contrast, analysis of carotenoid content and composition demonstrated that the white Marsh grapefruit is almost devoid of carotenoids (Figs. 2, 3 and 4), thus enabling a suitable comparison to analyse the physiological and molecular mechanism underlying the differences in carotenoid complement. Comparative HPLC-PDA analysis showed that green flavedo of both grapefruits contained similar concentration of the carotenoids characteristic of chloroplastic tissue (Fig. 3). However, it is noticeable that at the green stages Star Ruby contained higher α- and β-carotene content than Marsh (Fig. 3), indicating that the cyclization of lycopene to the ε,β- and β,β-branch of the pathway is operative at these stages of fruit development. This process is probably mediated by *ε-LCY* and *β-LCY1* genes that are expressed at relatively high levels in the flavedo of immature green fruits of both genotypes (Fig. 6). At the onset of fruit colouration after the mature-green stage, carotenoid content dramatically decreased in the flavedo and pulp of Marsh grapefruit with a profiling almost lacking of carotenoids (Figs. 3 and 4), in well agreement with previous results (Yokoyama and White, 1967; Banet et al. 1981; Xu et al. 2006a; Matsumoto et al. 2007). Analysis of the expression of genes of the MEP pathway (*DXS*, *HDS* and *HDR*; Fig. 5) and those of the early desaturation and cyclization of carotenes (Fig. 6) revealed virtual absence of important differences between Marsh and Star Ruby grapefruit. These results suggest that alteration in the pattern of transcription of these genes appears not to be the main factor responsible for the low carotenoid level in the white grapefruit. It has been proposed that this deficiency in carotenoids may be related to a deficient desaturation of phytofluene (Yokoyama and White 1967; Romojaro et al. 1979; Banet et al. 1981) or lower *PSY* activity (Costa et al. 2011). However, the alteration in carotenoid accumulation in the white Marsh grapefruit does not affect end-downstream metabolites of the pathway since ABA content substantially increased with fruit maturation in the flavedo and pulp of this genotype reaching concentrations similar to that found in other coloured *Citrus* fruits (Aung et al. 1991; Lafuente et al. 1997; Rodrigo et al. 2006). It is then reasonable to suggest different possibilities compatible with the data of this study and other results (Xu et al. 2006a), to explain the carotenoid complement in white grapefruits. First, a defect in the availability of substrates form early plastidic isoprenoid pathway that would reduce the flux to carotenoids. Second, metabolic processes related to carotenoid sequestration or aggregation may be also affected, reducing the capability to form complexes or to accumulate carotenoids in specific structures. For example, Or gene alters the formation of carotenoid-containing structures in pigmented cauliflower mutant (Lu et al. 2006), without affecting expression of carotenogenic pathway (Li et al. 2006). A third possibility would be related to an enhanced enzymatic degradation of carotenoids by carotenoid cleavage dioxygenases, as has been reported for white flower of chrysanthemum mutants (Ohmiya et al. 2006) or a white-fleshed peach mutant (Brandi et al. 2011).

Total carotenoid content (determined as β-carotene equivalent) in the red Star Ruby was higher in the pulp than in the flavedo (Fig. 2e–f). However, analysis of carotenoid composition revealed important concentrations of phytoene and phytofluene (Fig. 3), which were not accounted when measured at 450 nm. Then, carotenoid content, expressed as the sum of individual carotenoids, in the flavedo of Star Ruby grapefruit was twice than of the pulp, while in other orange-coloured citrus fruits ranged from seven to ten times higher in the flavedo than in the pulp (Gross 1987). Moreover, lycopene was the main carotenoid, followed by phytoene and phytoflene, accounting for more than 75 % of total carotenoids in the pulp (Fig. 4). It is interesting to remark that after colour break, xanthophylls were not

detected in the pulp of Star Ruby and the proportion of these carotenoids in the flavedo was around 2 % (Fig. 3). These results are in good agreement with previous data reported in mature fruit (Xu et al. 2006a; Fanciullino et al. 2008) and reinforce the motion of an independent regulation of carotenogenesis in the flavedo and pulp of *Citrus* fruit (Alquezar et al. 2008b; Matsumoto et al. 2009). Comparison of the differences in the carotenoid complements between both grapefruit indicates a partial blockage in the cyclization of lycopene in the red grapefruit, originating accumulation of early linear carotenes (phytoene, phytofluene and lycopene) and a reduction of xanthophylls. Analysis of ABA content in flavedo and pulp of Star Ruby grapefruit allowed us to support this assumption. Hormone concentration increased after colour break in the flavedo, but the levels reached in mature lycopene-accumulating fruits were about half of those in white grapefruit (Fig. 8). On the other hand, in the pulp, the pattern of ABA accumulation was different since ABA increased earlier and its content in Star Ruby was two to three times lower than in the pulp of Marsh (Fig. 8). With the exception of *NCED2* gene in the pulp, expression of NCEDs, key regulatory genes for ABA biosynthesis in *Citrus* fruits (Rodrigo et al. 2006; Kato et al. 2006; Alquezar et al. 2008b), followed a pattern consistent with that of ABA accumulation (Fig. 8). These results corroborate that the mutation in the tissues of the red grapefruit is more likely a partial blockage in the pathway, at the conversion of lycopene to β-carotene, which originates a restriction in the flux to downstream carotenoids (Alquezar et al. 2009; Mendes et al. 2011).

Analysis of the expression of key genes of the MEP pathway (*DXS*, *HDS* and *HDR*) and structural carotenoid biosynthetic genes in flavedo and pulp during fruit maturation of both genotypes only revealed significant differences in the expression of the chromoplast-specific *β-LCY2* gene (Figs. 5 and 6). If activities of MEP-related enzymes are paralleled with the pattern of gene expression, our data do not support the proposal that the flux of IPP precursors for carotenoids synthesis is stimulated in the red grapefruit, as has been suggested in the red-fleshed orange Cara Cara (Alquezar et al. 2008b). Moreover, expression profiling of these genes in flavedo and pulp of red and white grapefruits was similar to that previously reported for oranges and mandarins (Alós et al. 2006; Fanciullino et al. 2008; Alquezar et al. 2008b).

Expression of most carotenoid biosynthetic genes was much higher in the flavedo than in the pulp of both grapefruits and increased during fruit maturation except those of the ε, β-branch of the pathway that decreased coincident with the disappearance of ε, β-xanthophylls (Fig. 6), similarly to that reported in orange-coloured fruits (Kato et al. 2004; Rodrigo et al. 2004; Alós et al. 2006; Rodrigo and Zacarías 2006; Alquezar et al. 2008b). Costa et al. (2011) have described a reduced expression of *PSY* and *ZDS* genes in the albedo and pulp of the red Flame grapefruit as compared with Marsh. Our results only showed moderated differences in the expression of these two genes, but potential discrepancies with previous work (Costa et al. 2011) may be due to the different tissues analysed and the low capability to accumulate lycopene in the pulp of Flame grapefruit.

In the cyclization of lycopene to β-carotene in *Citrus* participates two genes, *β-LCY1* and *β-LCY2*, which have been demonstrated to have different in vitro activity and patterns of expression (Alquezar et al. 2009; Zhang et al. 2012). Moreover, the *β-LCY2* gene displayed a chromoplast-specific expression, and two alleles with marked differences in the ability to cyclizate lycopene have been reported (Alquezar et al. 2009). These evidences point to *β*-LCY2 as a key enzyme in the regulation of xanthophylls synthesis in *Citrus* fruits and potentially responsible for the accumulation of lycopene in red grapefruits (Alquezar et al. 2009; Mendes et al. 2011). Results from this study also demonstrated, by Northern blot analysis and qRT-PCR, that the expression of the chromoplast-specific *β-LCY2*, but not *β-LCY1*, was reduced in the pulp of the Star Ruby grapefruit as compared with that of the white genotype (Figs. 6 and 7). Despite these differences in the pulp of both grapefruits, it should be remarked that the expression of the *β-LCY2* gene in the pulp of Navel oranges, at a similar maturation stage, was about five times higher than in grapefruits and that the relative proportion of the two alleles was 70 % for *β-LCY2a* and 30 % for the *β-LCY2b*, of lower activity (Alquezar et al. 2009). On the other hand, in the pulp of both grapefruits, the proportion of the expression of the allele of higher activity was always below 45 %. In a recent phylogenetic analysis of the relationships among *Citrus* species, Garcia-Lor et al. (2013) concluded that allelic variations in the LCY loci should limit this biosynthetic step in pummelos. Our results reinforce and extend the proposal that a reduced expression of the *β-LCY2* gene and the altered ratio between the two alleles appears to be a common characteristic of *C. paradisi*. By contrast, the data suggest that oranges (Alquezar et al. 2009) and mandarins (Zhang et al. 2012) have an enhanced expression of the gene *β-LCY2* and also of the allele with higher activity (*β-LCY2a*) providing then a more efficient mechanism to convert lycopene in downstream xanthophylls.

Quantitative differences in the expression of the *β-LCY2* gene between white and red grapefruit (Fig. 7) may help to explain the biochemical data of lycopene accumulation. Carotenoid content in the white grapefruit is extremely low, and it is likely that the relatively high and constant expression of *β-LCY1* and the moderated increment of *β-LCY2* may be sufficient to allow an efficient conversion of lycopene to β-carotene and downstream xanthophylls. In the flavedo of the red grapefruit, the expression of *β-LCY2* also experienced an important increment during ripening,

and it was higher than in the pulp. Therefore, red colouration and accumulation of lycopene in the flavedo were not as noticeable as in the pulp. In this latter tissue, the expression of the *β-LCY2* is more severely impaired and should impose a blockage conducing to the accumulation of lycopene and other upstream carotenes (phytoene and phytofluene) and to the reduction in xanthophylls and ABA (Figs. 4 and 8). Other biochemical evidences support these assumptions. First, it is long time known that application of 2-(4-Chlorophenylthio) triethylamine hydrochloride, an inhibitor of lycopene cyclase activity, to white Marsh grapefruit induced an intense red colouration in the flavedo and a massive increment of carotenoids (40–46-fold increment), mostly lycopene and other upstream carotenes with respect to untreated fruits (Yokoyama et al. 1972), resembling the red grapefruit phenotype. Moreover, lycopene cyclization is reduced at temperatures above 30 °C, and Star Ruby grapefruits develop an intense and uniform red colour in the flavedo of fruits growing under warm climates around these temperatures (Tomes et al. 1956; Ladaniya 2008).

In summary, our results indicate that despite the white Marsh grapefruit is almost devoid of carotenoids in both flavedo and pulp, it is able to accumulate downstream products, like ABA, as in other citrus fruits, indicating that the carotenoid biosynthetic pathway is functional and other factors may be responsible for the reduced accumulation of carotenoids. Reduced expression of the chromoplast-specific *β-LCY2* gene and altered proportion in the expression of the allele with the high (*β-LCY2a*) and low (*β-LCY2b*) activity, with respect to orange-coloured fruits, appear to be a common feature in at least the two *C. paradisi* varieties analysed. Moreover, the expression of the *β-LCY2* gene was additionally reduced in the pulp of the red grapefruit as compared with the white, and this feature is likely responsible for the accumulation of lycopene in the red Star Ruby grapefruit.

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