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Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara

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ABSTRACT

Cara Cara is a spontaneous bud mutation of Navel orange (*Citrus. sinensis* L. Osbeck) characterized by developing fruits with a pulp of bright red coloration due to the presence of lycopene. Peel of mutant fruits is however orange and indistinguishable from its parental. To elucidate the basis of lycopene accumulation in Cara Cara, we analyzed carotenoid profile and expression of three isoprenoid and nine carotenoid genes in flavedo and pulp of Cara Cara and Navel fruits throughout development and maturation. The pulp of the mutant accumulated high amounts of lycopene, but also phytoene and phytofluene, from early developmental stages. The peel of Cara Cara also accumulated phytoene and phytofluene. The expression of isoprenoid genes and of carotenoid biosynthetic genes downstream *PDS* (phytoene desaturase) was higher in the pulp of Cara Cara than in Navel. Not important differences in the expression of these genes were observed between the peel of both oranges. Moreover, the content of the plant hormone ABA (abscisic acid) was lower in the pulp of Cara Cara, but the expression of two genes involved in its biosynthesis was higher. The results suggest that an altered carotenoid composition may conduct to a positive feedback regulatory mechanism of carotenoid biosynthesis in citrus fruits. Increased levels of isoprenoid precursors in the mutant that could be channeled to carotenoid biosynthesis may be related to the red-fleshed phenotype of Cara Cara.

1. Introduction

Carotenoids are a large family of isoprenoid compounds which provide attractive coloration to flowers and fruits, ranging from yellow, orange to deep red (Hirschberg, 2001; DellaPenna and Pogson, 2006). Their importance as nutritional components is also well recognized, since specific carotenoids are precursors of vitamin A, have significant antioxidant activity and protective effects against cardiovascular diseases and carcinogenesis, and from an industrial perspective (Clinton, 1998; Sandmann, 2001; Fraser and Bramley, 2004).

Carotenoids are the pigments responsible for the external and internal coloration of fruit of most citrus species; therefore, their content and composition have a strong impact in both the commercial and nutritional quality of the fruit. In past decades, carotenoid content and composition in the peel and pulp of fruit of different citrus cultivars were extensively studied (Gross, 1987; Xu et al., 2006a; Fanciullino et al., 2006; Matsumoto et al., 2007). Recently, genes coding enzymes of the main steps of the carotenoid biosynthetic pathway have been identified and their expression studied in the peel and pulp of different citrus species during natural (Kato et al., 2004; Rodrigo et al., 2004) or ethylene-induced (Rodrigo and Zacarias, 2007) fruit ripening. A schematic representation of the biosynthetic pathway of carotenoids is shown in Fig. 1. The peel of immature citrus fruit shows a carotenoid profile characteristic of chloroplast-containing tissue, being lutein (ε,β-xanthophyll) the main carotenoid. A noticeable decrease in lutein occurs at the onset of fruit coloration, with a parallel accumulation of specific β,β-xanthophylls, being 9-*Z*-violaxanthin the main carotenoid in the peel and pulp of mature orange-colored fruit, as oranges and mandarins. The massive increase in total carotenoids and β,β-xanthophylls occurring in the peel of orange and mandarin fruits during the transition from chloroplast to chromoplast is concomitant with the 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). The shift from the ε,β-branch to the β,β-branch of the pathway is also coordinated with a decrease in the expression of the lycopene ε-cyclase (ε-*LCY*) gene and, in the case of mandarin fruit, with an up-regulation of lycopene β-cyclase (β-*LCY*) gene (Kato et al., 2004; Rodrigo et al., 2004). In the pulp of fruit of most citrus species, total carotenoid content is usually lower than in the peel (Gross, 1987; Xu et al., 2006a), according to the lower expression of carotenoid biosynthetic genes in the pulp than in the peel (Kato et al., 2004). Collectively, these results suggest that carotenoid accumulation and composition during citrus fruit maturation is highly regulated by the coordinated transcriptional

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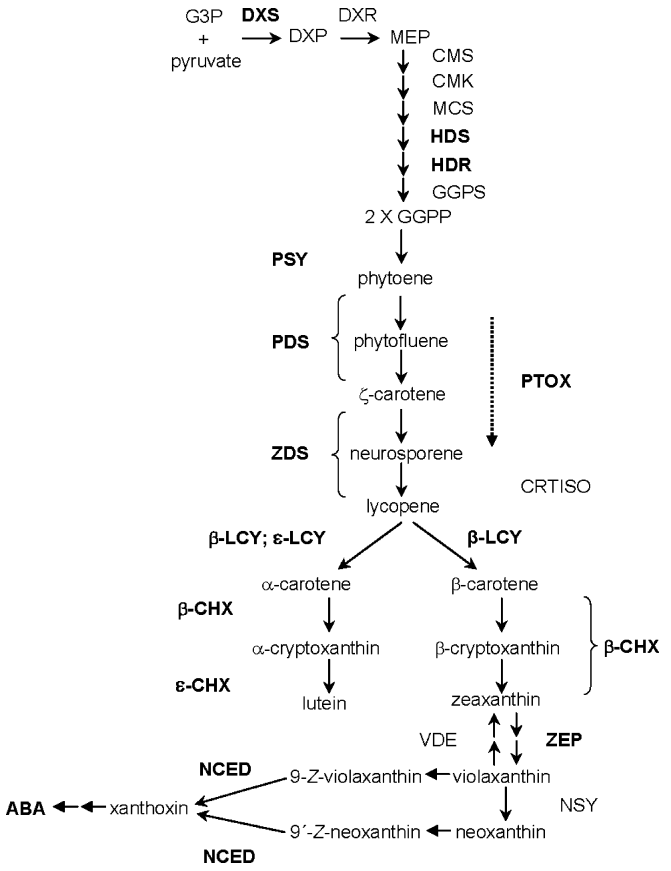

Fig. 1. Schematic diagram of the carotenoid biosynthesis pathway in plants. Genes which mRNA accumulation has been analyzed in this study are bold-lettered. G-3-P, D-glyceraldehyde 3-phosphate; *DXP*, 1-deoxy-D-xylulose 5-phosphate-synthase; DXP, 1-deoxy-D-xylulose 5-phosphate; *DXR*, DXP reductoisomerase; MEP, 2-*C*-methyl- D-erythritol 4-phosphate; *CMS*, 4-diphosphocytidyl-methylerythritol synthase; *CMK*, 4-diphosphocytidyl-methylerythritol kinase; *MCS*, methylerythritol 2,4-cyclodiphosphate synthase; *HDS*, hydroxymethylbutenyl 4-diphosphate synthase; *HDR*, hydroxymethylbutenyl 4-diphosphate reductase; *GGPS*, geranylgeranyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, ζ-carotene desaturase; *PTOX*, plastid terminal oxidase; *CRTISO*, carotene isomerase; *ε-LCY*, lycopene ε-cyclase; *β-LCY*, lycopene β-cyclase; *β-CHX*, β-carotene hydroxylase; *ε-CHX*, ε-carotene hydroxylase; *ZEP*, zeaxanthin epoxidase; *VDE*, violaxanthin de-epoxidase; *NSY*, neoxanthin synthase; *NCED*, nine-*cis*-epoxycarotenoid dioxygenase; ABA, abscisic acid.

activation of carotenoid biosynthetic genes, as it has been demonstrated in other fruits (Hirschberg, 2001; Fraser and Bramley, 2004). In addition to the carotenoid biosynthetic genes, other upstream metabolic pathways in the plastids, as 2- *C*-methyl-D-erythritol 4-phosphate (MEP) pathway, have been demonstrated to influence carotenoid content and composition in fruit of different species (Rodriguez-Concepcion and Boronat, 2002). During tomato fruit ripening, a correlation between the expression of 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) and hydroymethylbutenyl diphosphate reductase (*HDR*) genes and carotenoid accumulation has been reported (Lois et al., 2000; Botella-Pavia et al., 2004).

Lycopene accumulation is an unusual feature in most citrus fruits, since has only been reported in few species. Most of the lycopene-accumulating mutants have been identified in grapefruit (*Citrus paradisi* Macf.) and pummelo (*Citrus grandis* Osbeck) (Saunt, 2000), an only three in oranges (*Citrus sinensis* L. Osbeck): Shara (Monselise and Halevy, 1961), Cara Cara (Saunt, 2000) and the recently characterized Hong Anliu (Liu et al., 2007). Cara Cara was originated in Venezuela as a bud mutation from the Washington Navel orange and its pulp resembles that of pink and red grapefruits because of the bright red coloration, while the external fruit color remains orange. Carotenoid composition in the juice of mature Cara Cara fruit showed that its peculiar pulp coloration is due to lycopene accumulation, which is completely absent in the parental orange (Lee, 2001). In addition, pulp of mature Cara Cara fruit accumulates unusually high amounts of early linear carotenes (phytoene and phytofluene) and β-carotene (Lee, 2001; Xu et al., 2006a). The flavedo of mature Cara Cara fruits also accumulates high amounts of phytoene but not lycopene, suggesting that this tissue may be also affected by the mutation (Xu et al., 2006a). Xu et al. (2006a) suggested that the biochemical basis of lycopene accumulation in Cara Cara might affect early isoprene pathway or phytoene synthase, the first specific step of carotenoid biosynthesis. However, accumulation of *PSY* transcripts was found to be similar in pulp of both Cara Cara and ordinary Navel oranges (Tao et al., 2007). Therefore, the objective of this study was to elucidate the relationship between the biochemical changes leading to lycopene accumulation in Cara Cara fruit and the expression of isoprenoid and carotenoid biosynthetic genes. To that end, we analyzed carotenoid content and composition in the peel and pulp of Cara Cara and Navel oranges during development and maturation. We also studied the expression of three genes of the MEP pathway (*DXS, HDS* and *HDR)* and nine genes of the carotenoid biosynthetic pathway involved in early condensation and desaturation reactions (*PSY, PDS, ZDS*), coupled redox reactions (*PTOX*), cyclizations (*ε-LCY* and *β-LCY*), hydroxylations (*ε-CHX* and *β-CHX*) and epoxidation (*ZEP)* in tissues of both genotypes. The results of this study were the first, to our knowledge, to provide information comparing the profiles of carotenoid accumulation and the expression of isoprenoid and carotenogenic genes in fruits of the red mutant Cara Cara and its parental variety during maturation.

The plant hormone abscisic acid (ABA) is an end product of the carotenoid biosynthetic pathway generated by the enzymatic cleavage of either 9-*Z*-violaxanthin or 9′-*Z*-neoxanthin (Schwartz et al., 2003). The enzyme responsible for this cleavage is referred to as nine-*cis*-epoxycarotenoid dioxygenase (NCED). In citrus, two *NCEDs* genes have been recently isolated and analysis of their expression revealed a differential regulation during fruit development and maturation, by ethylene or under stress conditions (Rodrigo et al., 2006). Accumulation of ABA is severely reduced in some of the carotenoid deficient mutants identified in different plant species, as maize (Hable et al., 1998), *Arabidopsis* (Rock and Zeevaart, 1991; Tian et al., 2004) and citrus (Rodrigo et al., 2003). To determine if ABA content is impaired in the lycopene-accumulating mutant Cara Cara, ABA levels and expression of both *NCED1* and *NCED2* genes were analyzed in the peel and pulp of Cara Cara during fruit development and maturation and compared with those of the parental Navel.

2. Results

2.1. Fruit growth, color and chlorophyll and carotenoid content in Navel and the red-fleshed Cara Cara oranges during development and maturation

Evolution of fruit growth, measured as fruit diameter, fruit color, and chlorophyll and carotenoid content was analyzed in Navel and Cara Cara fruits harvested at ten developmental/maturation stages (Figs. 2 and 3). Changes in fruit growth and external color were similar for both Navel and Cara Cara oranges throughout the whole development and maturation process (Figs. 2 and 3). However, important differences in pulp coloration between Navel and Cara Cara fruits were observed from early stages of fruit development. The pulp of immature-green fruits of Navel from middle June presented a greenish-yellow color while that of Cara Cara showed a distinctive reddish-pink color (Fig. 2). Then, as fruits of

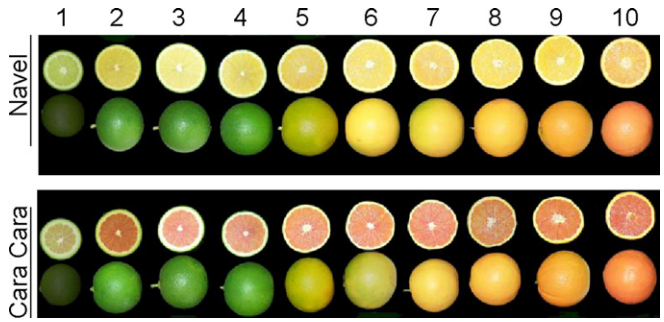

Fig. 2. External and internal appearance of Navel and Cara Cara fruits during development and maturation. Numbers from 1 to 10 correspond to the sampling stages used for the experiments, and numbers 2, 4, 6 and 10 correspond to the IG (Immature-green), MG (Mature-green), B (breaker) and FC (Full-color) stages, respectively. Picture corresponds to fruits harvested in season 2004/2005.

Navel orange matured, the pulp turned from a yellowish-orange to a characteristic orange color. The flesh of Cara Cara remained deep red during the whole development and maturation, showing orange shades in advanced mature fruits (Fig. 2). As a result of these changes, the *a* Hunter parameter (which develop from negative to positive values as the color change from green to red) and the *a/b* ratio were higher in the flesh of Cara Cara than in that of Navel (Fig. 3A and B).

As expected by the rate of fruit degreening, no important differences in the rate and the time of chlorophyll disappearance were found in the peel of both orange varieties (Fig. 3C and D). Total carotenoid content (expressed as μg of β-carotene equivalents) was similar in the peel of Navel and Cara Cara oranges during development and maturation (Fig. 3C and D). The increase in total carotenoid started at the same time (late November) and reached a similar maximum in the peel of fruits of both varieties (Fig. 3C and D). In the pulp of Cara Cara fruit, however, the evolution of carotenoid content was markedly different to that of Navel. In the parental variety, carotenoid content in the pulp was very low until the breaker stage (middle November), then increased rapidly to reach the highest value at early December and decreased thereafter (Fig. 3E). Accumulation of total carotenoids in the pulp of Cara

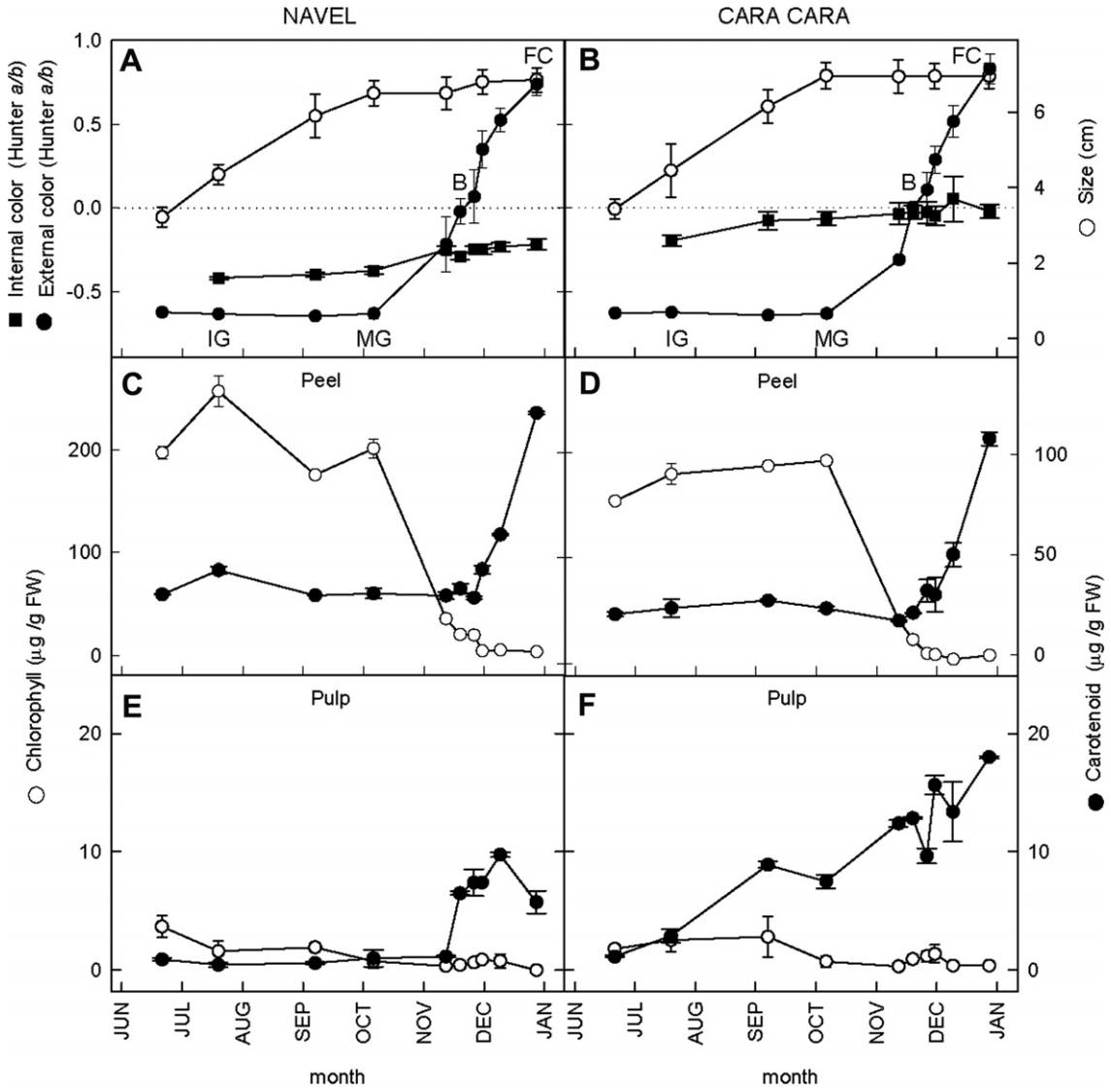

Fig. 3. Evolution of fruit diameter and peel and pulp color (A and B) and chlorophyll and carotenoid contents in the peel (C, D) and in the pulp (E, F) during development and maturation of Navel (A, C, E) and Cara Cara (B, D, F) fruits (*C. sinensis* L. Osbeck). The 10 samples analyzed correspond to the developmental and maturation stages indicated in Fig. 2. Fruit color is expressed as the *a/b* Hunter ratio. The dotted line indicates the color index at the color break. The physiological fruit stages IG (Immature-green), MG (Mature-green), B (breaker) and FC (Full-color) are indicated. The data are means ± SD of at least three independent measurements.

Cara fruit was evident from early stages of development (mid July) and steadily increased throughout the whole maturation process. The pulp of immature-green fruit of Cara Cara contained 6-times more carotenoids than the corresponding tissue of Navel fruit. From the breaker stage to full maturation, total carotenoid content in pulp of Cara Cara was 2- to 3-times higher than in the pulp of Navel (Fig. 3E and F).

2.2. Carotenoid composition during fruit development and maturation in flavedo and pulp of Navel and the red-fleshed mutant Cara Cara orange

The composition of carotenoids was analyzed in flavedo and pulp of Navel and Cara Cara oranges at four developmental/ripening stages: immature-green (IM), mature-green (MG), breaker (B) and full-colored (FC) fruits (Fig. 2). By HPLC-PDA analysis twelve major carotenoids were quantified, accounting for more than 90% of total carotenoids in all samples analyzed. Flavedo of green fruits, either IG or MG, from both varieties presented a carotenoid profile characteristic of chloroplastic tissue, being lutein the predominant carotenoid (Fig. 4). However, in green flavedo of Cara Cara fruit, the content of linear carotenes (phytoene, phytofluene and lycopene) was higher than in the corresponding tissue of Navel fruit. The content of phytoene, per example, was 14- and 4-times higher in the mutant at IG and MG stages, respectively. On the other hand, a reduction in xanthophyll content (mainly lutein and neoxanthin) was detected in the peel of Cara Cara fruits at the IG stage. In the flavedo of fruits of both varieties at the B stage, the content of ε,β-xanthophylls decreased and β,β-xanthophylls increased, being phytoene and 9-*Z*-violaxanthin the most abundant carotenoids. The peel of mutant fruits at this stage contained a low concentration of lycopene (less than 100 ng/g fr. wt), which was absent in Navel orange. At FC stage, the most remarkable difference in carotenoid composition between Navel and Cara Cara flavedo was the important accumulation of phytoene in the mutant, which was more than 5-times higher that in parental fruits. The distribution and content of other components were similar in the peel of mature fruits of both varieties (Fig. 4).

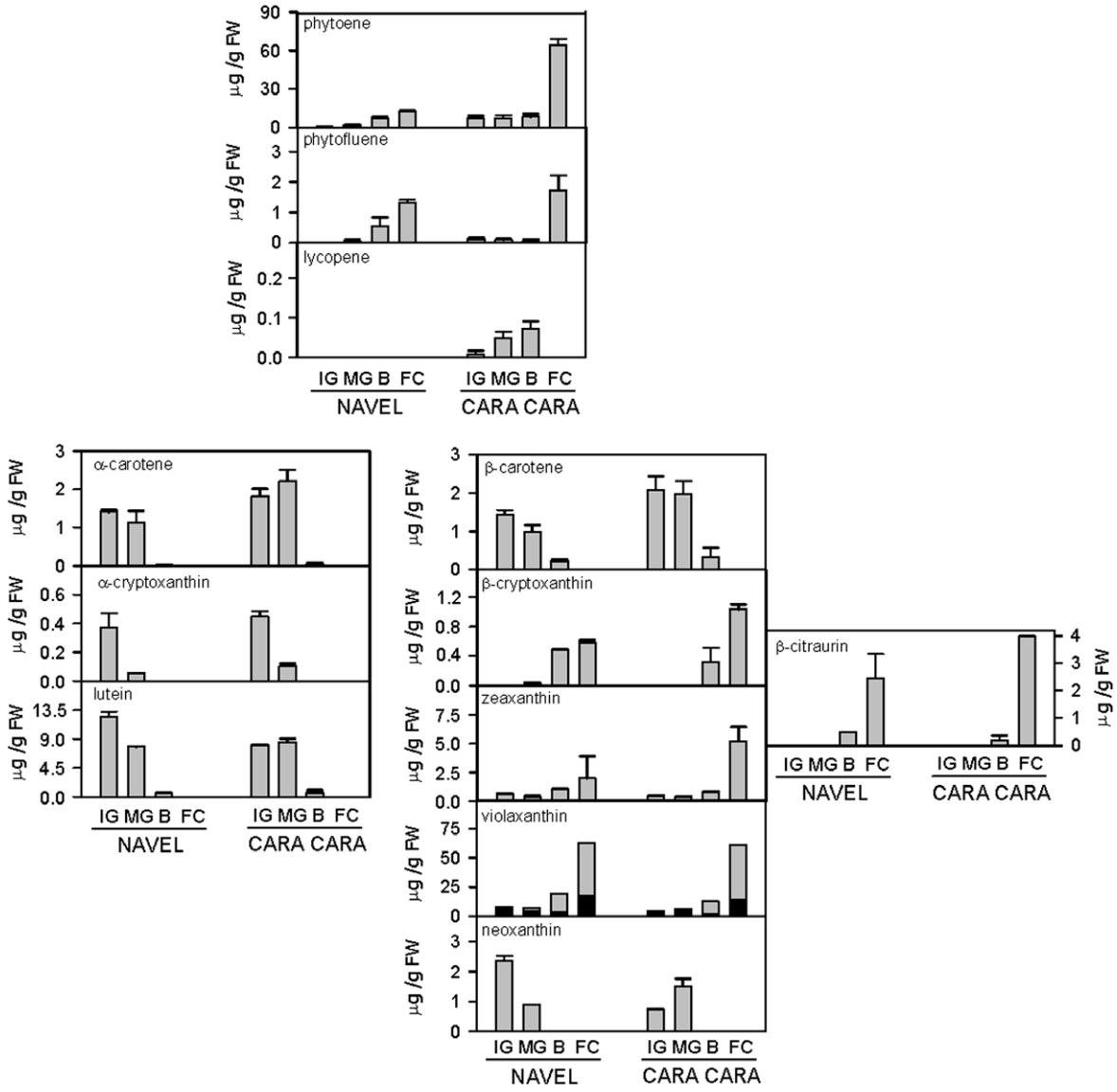

Fig. 4. Carotenoid composition in the peel of Navel and Cara Cara fruits (*C. sinensis* L. Osbeck) at the IG (Immature-green), MG (Mature-green), B (breaker) and FC (Full-color) stages. The plots were arranged following the carotenoid biosynthetic sequence in the pathway. When *E*- and *Z*-isomers of a particular carotenoid are identified, the *Z*-isomer is represented in grey color. The data are means ± SD of at least three independent measurements.

Evolution of carotenoid content and composition in the pulp of Cara Cara oranges was markedly different to that of Navel, and differences were evident from the IG stage, well before the initiation of the maturation process (Fig. 5). During maturation of Navel orange, the pulp accumulated almost exclusively β,β-xanthophylls, being 9-*Z*-violaxanthin the major carotenoid, which accounted for more than 90% of total carotenoids in mature fruits. By contrast, linear carotenes were the predominant carotenoids in the pulp of the red-fleshed mutant, accounting for more than 94% of total carotenoids at all stages analyzed. The concentration of phytoene increased progressively as fruit matured and the pulp of FC Cara Cara fruit contained 50-times more phytoene than the pulp of Navel oranges. Phytofluene, lycopene and β-carotene, which were not detected in Navel orange throughout the whole maturation, also accumulated in Cara Cara pulp from early stages of fruit development. Accumulation of β,β-xanthophylls was delayed in the pulp of mutant fruit, but at full maturation both varieties reached similar concentration of these components (Fig. 5), which explains the orange shadows observed in the red background of Cara Cara pulp (see stage 10 of Fig. 2).

It is interesting to note that colorless carotenes were not quantified when total carotenoid content was determined as μg of β-carotene equivalents (Fig. 3), due to the different maximum absorption wavelength between β-carotene (452 nm), and phytoene (285 nm) and phytofluene (345 nm). Therefore, considering the important amount of colorless carotenes in Cara Cara fruit tissues, total carotenoid content was recalculated in all the samples analyzed by HPLC-PDA as the sum of individual carotenoids. From these analyses, differences in total carotenoid content between peel and pulp of Cara Cara fruit and Navel oranges were even higher. Flavedo of Cara Cara fruit at FC stage accumulated 70% more carotenoids than that of Navel, and in the pulp the differences ranged from 20- at B stage to 170-times at MG stage.

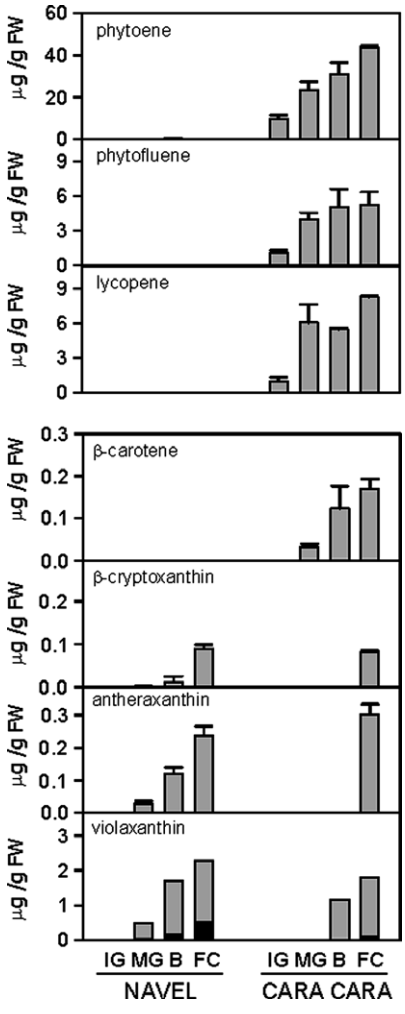

Fig. 5. Carotenoid composition in the pulp of Navel and Cara Cara fruits (*C. sinensis* L. Osbeck) at the IG (Immature-green), MG (Mature-green), B (breaker) and FC (Full-color) stages. The plots were arranged following the carotenoid biosynthetic sequence in the pathway. When *E*- and *Z*-isomers of a particular carotenoid are identified, the *Z*-isomer is represented in grey color. The data are means ± SD of at least three independent measurements.

2.3. Comparison of the expression of isoprenoid and carotenoid biosynthetic genes in flavedo and pulp of fruits of Navel and the red-fleshed mutant Cara Cara orange

To determine whether the unusual carotenoid composition in Cara Cara fruit is related to altered gene expression, accumulation of transcripts corresponding to three genes of the MEP pathway and nine genes of the carotenoid pathway was analyzed in the flavedo and pulp of Cara Cara and Navel fruits at four developmental/ripening stages. It should be mentioned that for all the genes and developmental stages analyzed, accumulation of the transcripts was always higher in flavedo than in pulp (Figs. 5 and 6). The three genes related to the MEP pathway showed an expression pattern in the peel different to that of carotenoid biosynthetic genes. Accumulation of *DXS* and *HDR* mRNAs showed a maximum at the MG stage, decreasing at the B stage and remained constant thereafter. *HDS* transcript accumulated progressively in the flavedo throughout fruit development and ripening (Fig. 6). In the flavedo of IG and MG fruits, accumulation of these transcripts was slightly higher in Cara Cara oranges than in Navel. It is important to note that in the pulp the expression of the three MEP genes increased with fruit maturation. The hybridization signal for *HDS* at the four developmental stages analyzed was higher in Cara Cara pulp than in Navel. For *DXS* and *HDR*, the hybridization signals were also more abundant in the pulp of mutant fruit at B and FC stages (Fig. 6). From these results, it appears that the expression of genes of the MEP pathway was higher in the flesh of the lycopene-accumulating mutant Cara Cara.

In the flavedo and flesh of Navel orange, expression of most carotenoid biosynthetic genes was induced during fruit development and maturation. The exception to that pattern was the expression of the genes encoding enzymes exclusively involved in the formation of ε,β-xanthophylls, as ε-*LCY* and ε-*CHX*, which were down-regulated (Fig. 7). Comparison of transcript accumulation between Navel and Cara Cara revealed differences, in both the flavedo and pulp. Accumulation of *ZDS* and β-*LCY* mRNAs was slightly higher in the peel of Cara Cara than in Navel oranges, but not differences in transcripts accumulation of the remaining genes were observed in the flavedo of both varieties. In the pulp of Cara Cara, the expression of most carotenoid biosynthetic genes, except those for ε,β-xanthophylls, was higher than in Navel fruit, and this difference was more prominent as fruit maturation progressed. Analysis of β-*LCY* gene expression revealed a remarkable difference in the pattern and level of expression between Cara Cara and Navel pulp. In Navel pulp, β-*LCY* showed a constitutive expression during fruit ripening, while in Cara Cara its expression increased continuously, reaching at the FC stage the maximum hybridization signal, which was around 3-times higher than in Navel (Fig. 7). To ensure the differences in gene expression observed between the pulp of Cara Cara and Navel fruits, a qRT-PCR analysis were performed for genes which displayed most relevant variations. Fig. 8 shows that *HDS* mRNA accumulated to higher levels in the pulp of Cara Cara fruit throughout the whole maturation period, whereas those of *DXS* and β-*LCY* were also higher after the B and MG stage, respectively.

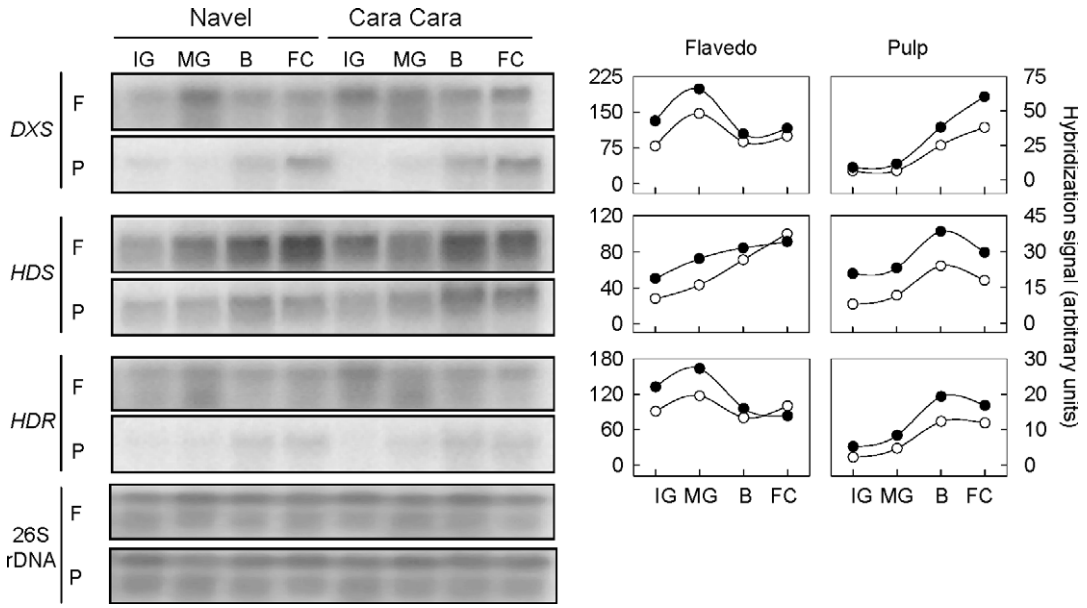

Fig. 6. Accumulation of mRNAs from MEP genes in the flavedo (F) and the pulp (P) of Navel (white symbols) and Cara Cara (black symbols) fruits (*C. sinensis* L. Osbeck) at the IG (Immature-green), MG (Mature-green), B (breaker) and FC (Full-color) stages. All transcripts values for individual genes were normalized with respect to the corresponding value of the 26s rRNA signal. Normalized values of mRNAs accumulation in arbitrary units are represented at the right, using the FC flavedo of Navel as a reference (100).

2.4. Comparison of ABA levels and the expression of NCED genes in flavedo and pulp of fruits of Navel and the red-fleshed mutant Cara Cara orange

ABA content and expression of *NCED* genes have been shown to be affected in carotenoid hydroxylation deficient mutants of different plant species, and a feedback regulation of ABA biosynthesis has been then proposed (Schwartz et al., 2003; Tian et al., 2004). Therefore, we postulated whether alterations in the carotenoid content and in the expression of carotenoid biosynthetic genes in Cara Cara fruit may have also affected ABA content and the expression of *NCEDs*, key genes of ABA biosynthesis. In the peel of green fruit of both genotypes, ABA content was very low and increased concomitantly with the transformation from chloroplast to chromoplast. The expression of *NCED1* and *NCED2* paralleled the increase in ABA content and was similar in the flavedo of Navel and Cara Cara oranges (Fig. 9). In the pulp, however, differences in the ABA content and in the expression of *NCEDs* genes were detected. The ABA content in the pulp was lower than in the flavedo, and whereas in the parental line ABA increased progressively with fruit maturation, in the mutant that increase was lower and delayed. The pulp of Cara Cara fruit at B and FC stages contained 77% and 36% less ABA than the corresponding tissue of Navel oranges. By contrast, the expression of *NCED1* and *NCED2* were higher in the pulp of the mutant, virtually at the four maturation stages (Fig. 9).

3. Discussion

In the present work we have studied the carotenoid biosynthetic pathway at molecular and biochemical level in fruits of Cara Cara mutant in order to understand the basis of its lycopene accumulation in the pulp. Changes in coloration and in total carotenoids in the pulp throughout fruit development and maturation revealed that the effect of the mutation was manifested from early stages of fruit development (immature-green fruit) and leading an early activation of carotenogenesis in the pulp (Fig. 3). As a consequence, the timing of carotenoids accumulation in the flesh of the mutant was also affected and initiated several months before that in the parental orange. The mutation, however, has only minor effects and later on development in the carotenoid complement of the peel. These differences in the timing and pattern of carotenoids accumulation between flavedo and pulp of Cara Cara orange reinforce the hypothesis of an independent regulation of carotenoid biosynthesis in these tissues, as previously suggested for citrus fruits (Kato et al., 2004; Xu et al., 2006a).

Comparison of the carotenoid profile and content in the peel and pulp of Cara Cara and Navel fruits revealed that the most remarkable feature in the mutant was not only the large concentration of lycopene in the pulp, but also the accumulation of higher amounts of colorless and bicyclic carotenes (Figs. 3 and 4). This carotenoid profile resembles that usually found in other lycopene-accumulating mutants, such as grapefruit and pummelo (Gross, 1987; Xu et al., 2006a). The pulp of red-fleshed grapefruit and pummelo mutants contained reduced amounts of xanthophylls, hardly reaching 5% of total carotenoids. Based on these carotenoid complements, it has been suggested for these mutants a defective β-carotene hydroxylation (Xu et al., 2006a) but the expression of carotenoid biosynthetic genes has not been examined in these mutants. In Cara Cara oranges, however, minor alterations in the concentration of downstream xanthophylls were found during the green stages. In spite of this, full mature fruit tissues (FC stage) of Cara Cara contained similar concentrations of xanthophylls than the corresponding tissues of Navel oranges, in agreement with previous data (Xu et al., 2006a). Taken together, these observations suggest that the mechanisms underlying lycopene accumulation in the red Cara Cara mutant are different to that operating in red grapefruits and pummelos (Xu et al., 2006a). A simple explanation for the phenotype of Cara Cara fruit would be a pulp-specific blockage in the conversion of lycopene to β-carotene. This hypothesis, however, is unlikely since total xanthophylls are not significantly affected in the flesh of Cara Cara fruits. Thus, the phenotype of Cara Cara pulp appears to be the result of a mutation superimposed over the ordinary carotenogenesis process from early stages of fruit development, originating accumulation of lycopene besides the normal carotenoid complement of the pulp.

The comparative analysis of the expression of three genes of the MEP pathway and nine carotenogenic genes in Navel and Cara Cara

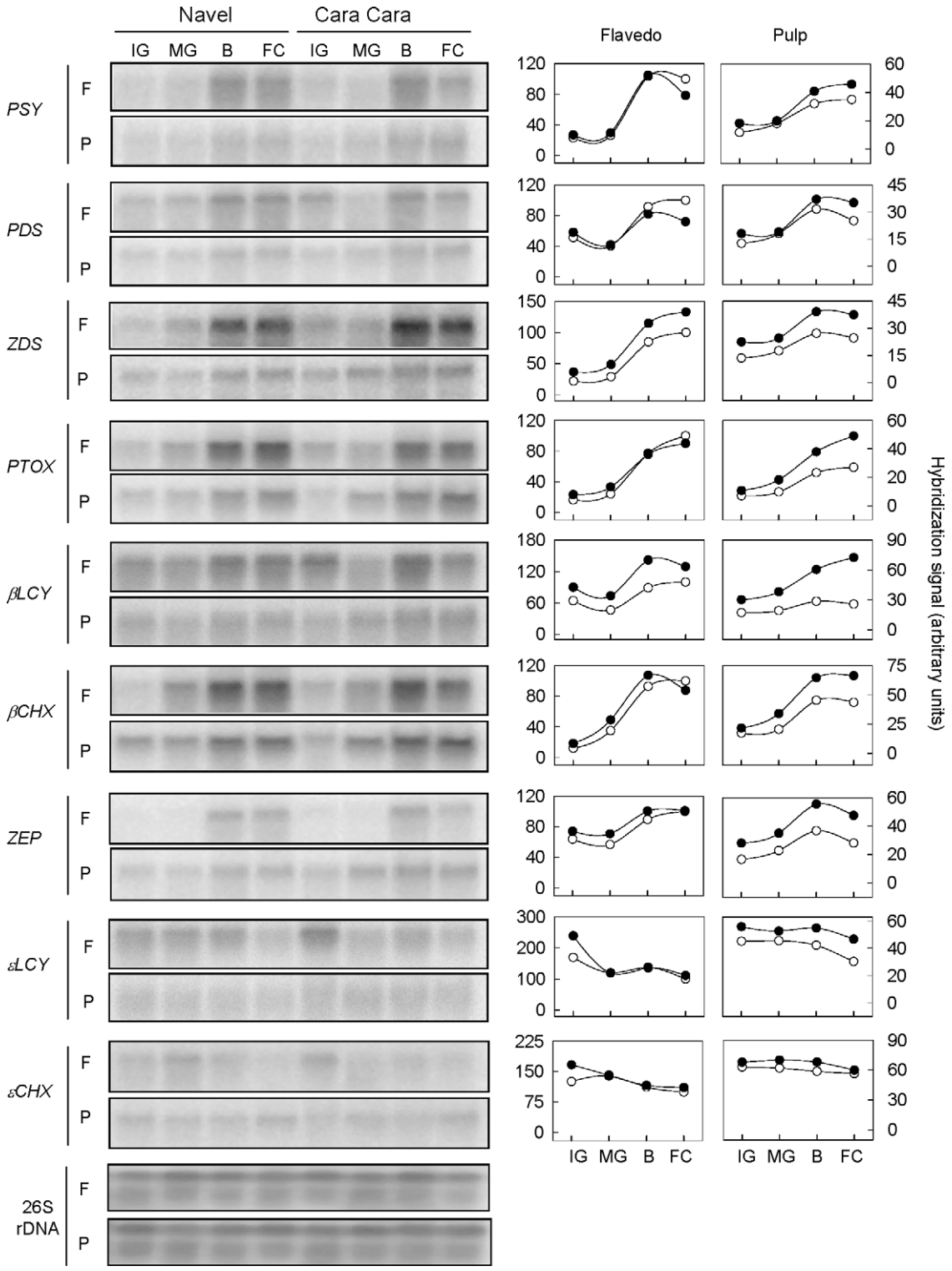

Fig. 7. Accumulation of mRNAs from carotenoid biosynthetic genes in the peel (F) and the pulp (P) of Navel (white symbols) and Cara Cara (black symbols) fruits (*C. sinensis* L. Osbeck) at the IG (Immature green), MG (Mature green), B (breaker) and FC (Full-color) stages. All transcripts values for individual 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 right, using the FC flavedo of Navel as a reference (100).

fruits tissues revealed important differences which were mainly ascribed to the pulp. Accumulation of the mRNAs corresponding to *DXS*, *HDS* and *HDR* was higher in the flavedo of green Cara Cara fruit as compared with those of Navel. In the flavedo of mandarin fruits (*Citrus clementina*) a similar expression for *DXS* has been observed (Alos et al., 2006). Interestingly, in the pulp of oranges, the expression of the three MEP genes was up-regulated concomitantly with the carotenogenic genes (Figs. 6–8 and Rodrigo et al., 2004), suggesting a role of this pathway in the production of precursors for the burst of carotenoid biosynthesis occurring during citrus fruit ripening. In the flesh of Cara Cara fruits the expression of the three MEP genes was higher than in the flesh of the parental

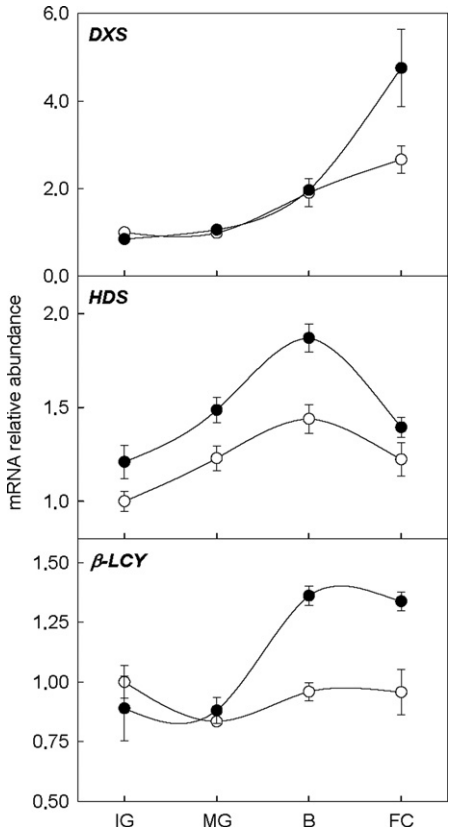

Fig. 8. Quantitative RT-PCR analysis of the expression of *DXS*, *HDS* and β-*LCY* genes in the pulp of Navel (white symbols) and Cara Cara (black symbols) fruits (*C. sinensis* L. Osbeck) at the IG (Immature green), MG (Mature green), B (breaker) and FC (Full-color) stages. The levels of expression were normalized to the amount of RNA and the value of Navel pulp at the IG stage was set to 1. The data are means ± SD of three experimental replicates.

line and these differences were especially prominent for *HDS* (Figs. 6 and 8). These differences were observed by semi-quantitative Northern blot analyses and confirmed by qRT-PCR. In tomato fruit, both *DXS* and *HDR* have been reported to be key control steps of the MEP pathway (Lois et al., 2000; Botella-Pavia et al., 2004), while *HDS* is constitutively expressed, and thus seems to play a non-limiting role in carotenoid biosynthesis during tomato ripening (Rodriguez-Concepcion et al., 2003). The fact that the expression of *HDS* gene was the only one of the MEP pathway up-regulated during maturation in both flavedo and pulp and in a similar fashion that other carotenogenic genes, suggests a key regulatory role for this gene in the production of isoprenoid precursors in orange fruits for the carotenoid burst. If the increase in *HDS* transcripts levels would result in similar changes in protein abundance and activity, it is conceivable that Cara Cara flesh may contain increased levels of isoprenoid precursors available for carotenoid biosynthesis from early stages of fruit development. As a consequence, the metabolic flux of the carotenoid pathway would be unbalanced and may originate accumulation of early carotenes, as we observed in the flesh of Cara Cara fruit (Fig. 5).

The expression of most of the carotenogenic and *NCED* genes were similar in flavedo of Navel and Cara Cara fruits (Figs. 7–9). The formation of phytoene, catalyzed by PSY, is thought to be a rate-limiting step in carotenoid biosynthesis (Giuliano et al., 1993; Bramley, 2002; Fraser et al., 2002). Concentration of phytoene in flavedo and pulp of Cara Cara at full mature stage was 5- and 50-times higher, respectively, than in Navel (Figs. 3 and 4). Nevertheless, the expression of *PSY* gene was similar in tissue of both oranges, suggesting that accumulation of phytoene in the mutant might be the result of an enhancement of upstream metabolic pathways. Recently, other orange mutant, Hong Anliu, which also accumulates lycopene in the pulp, has been characterized and an up-regulation of most carotenogenic genes has been proposed as the mechanism responsible for its particular carotenoid composition (Liu et al., 2007). But in contrast with the phenotype of Cara Cara, the pulp of Hong Anliu mutant fruit does not accumulate early carotenes such as phytoene and phytofluene (Liu et al., 2007).

It is interesting to comment that in the pulp of Cara Cara fruit, all genes examined downstream *PDS*, including *NCEDs*, showed higher expression levels than in the pulp of Navel. This general

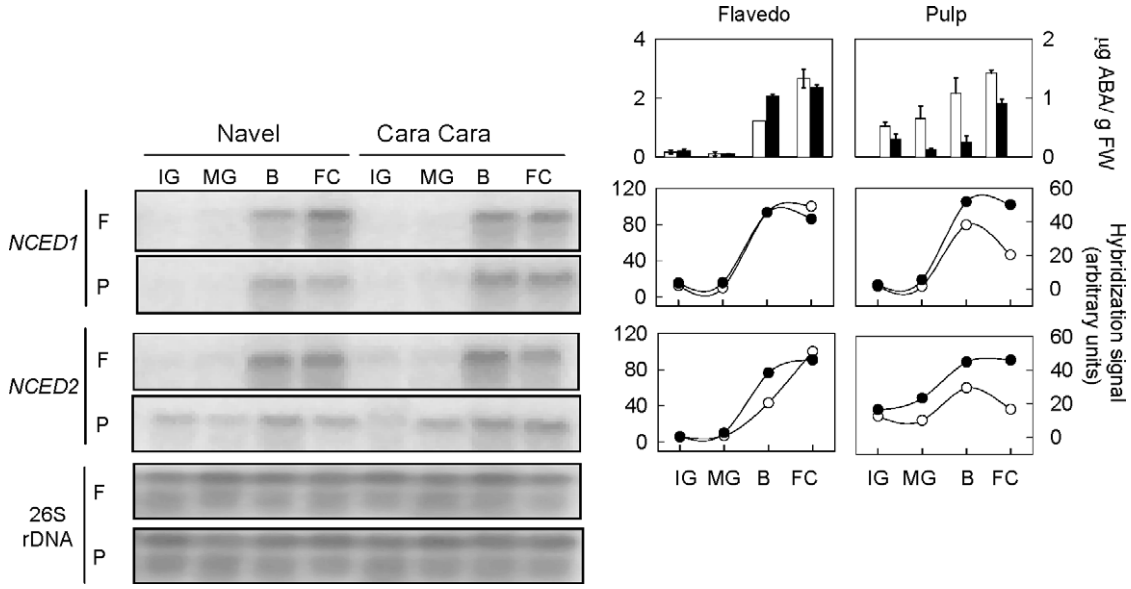

Fig. 9. ABA content in flavedo and pulp of Navel (white columns) and Cara Cara (black columns) fruits (*C. sinensis* L. Osbeck). Note the different ABA scale for each tissue. ABA values are the mean ± SD of al least three measurements. Accumulation of mRNAs from ABA biosynthetic genes in the peel (F) and the pulp (P) of Navel (white symbols) and Cara Cara (black symbols) fruits at the IG (Immature green), MG (Mature green), B (breaker) and FC (Full-color) 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 right, using the FC flavedo of Navel as a reference (100).

up-regulation of the carotenoid biosynthetic genes in the flesh of the mutant could be the result of a regulatory feedback mechanism induced by the increased levels of lycopene or/and β-carotene. Similar feedback regulatory mechanism in the carotenoid pathway has been reported in other plants (Giuliano et al., 1993; Corona et al., 1996; Al Babili et al., 1999; Ronen et al., 2000; Romer et al., 2000) and also suggested for citrus fruit (Xu et al., 2006b). Moreover, alterations in the levels of other upstream metabolites, as those of the MEP pathway, could also affect the expression of carotenoid biosynthetic genes in Cara Cara fruit. In tomato fruits, it has been shown that increased levels of 1-deoxy-D-xylulose stimulated the expression of *PSY1* and carotenoid content (Lois et al., 2000; Rodriguez-Concepcion, 2006).

Cyclization of lycopene by either ε- or β-lycopene cyclases, has been shown to play a key role in the regulation of carotenoid composition in fruit and flower of different plant species, as their relative expression may deviate the balance of the pathway to the formation of ε- or β-carotenoids (Cunningham, 2002; Bouvier et al., 2005). During ripening of citrus fruit, the coordinated down-regulation of ε-*LCY* and stimulation of β-*CHX* expression redirect the flux of the pathway into the β,β-branch, resulting in the massive accumulation of β,β-xanthophylls (Kato et al., 2004; Rodrigo et al., 2004). In tomato, up-regulation of early carotenoid biosynthetic genes and down-regulation of β- and ε-*LCY* leads to the accumulation of lycopene and early carotenes (Bramley, 2002). In Cara Cara we did not observed any significant alteration in the expression pattern of ε-*LCY* in the flavedo and pulp that could directly explain the phenotype observed. However, since a slight delay in the accumulation of β,β-xanthophylls was observed in the pulp of the mutant, it is tempting to speculate that a partial loss in β-LCY activity might be also occurring. The cyclization of lycopene is a limiting step in carotenoid biosynthesis thus, a minor alteration in the functionality of β-LCY could dramatically affect carotenoid composition, increasing the levels of lycopene and other upstream carotenoids and reducing those of xanthophylls. Interestingly, the expression of β-*LCY* was highly up-regulated in Cara Cara pulp during maturation while in Navel remained with minor changes. Even thought the expression of β-*LCY* was highly up-regulated in Cara Cara pulp, lycopene accumulated to high level during maturation, suggesting a putative lower activity of the enzyme in the mutant (Fig. 5), however, this hypothesis requires further characterization of the mutant. In addition, the induction of β-*LCY* transcription in the pulp of Cara Cara might be mediated by the high levels of lycopene accumulated from early stages of development. The stimulation of the β-*LCY* transcription by alterations in lycopene content has been previously described in CPTA-treated *Narcissus* flowers (Al Babili et al., 1999) and in transgenic tomato fruits with increased lycopene accumulation (Romer et al., 2000).

Biosynthesis of ABA in fruit of different species of citrus seems to be highly regulated by *NCEDs* and the level of expression of these genes has been shown to correlate with ABA content in the flavedo during natural and artificial fruit maturation (Rodrigo et al., 2006; Kato et al., 2006; Agusti et al., 2007). Our results indicate that alterations in the carotenoid content also affect expression of ABA biosynthetic genes and ABA content in citrus fruits (Fig. 9). In the pulp of mutant fruits, expression of *NCED* genes was higher than in the parental line but the increase in ABA content was lower and delayed. It should be mentioned that these alterations in ABA levels were in parallel with the changes in the concentration of 9-*Z*-violaxanthin (Fig. 5). These results suggest that the amount or availability of the epoxycarotenoid 9-*Z*-violaxanthin in the pulp of citrus fruit is a limiting factor for ABA biosynthesis. In carotenoid-deficient mutants of *Arabidopsis*, the reduction in violaxanthin and neoxanthin also resulted in lower drought-induced ABA content and *NCED3* gene expression, the rate-limiting step for ABA biosynthesis under water stress conditions (Tian et al., 2004). Alterations in ABA content in Cara Cara pulp appears not to be deleterious for normal fruit development and ripening, similarly to the observation in other citrus mutant, Pinalate, in which a fruit-specific blockage at the ζ-carotene desaturation also resulted in reduced ABA content (Rodrigo et al., 2003).

In conclusion, flavedo and pulp of the Cara Cara mutant appear to be affected by the mutation since both tissues contain higher levels of carotenes, but lycopene accumulation and red coloration occurred only in the pulp. A higher expression level of the MEP pathway genes was observed in the pulp of Cara Cara, and in particular *HDS*, suggesting an increased production and channeling of isoprenoid precursors into the carotenoid pathway that could result in an elevation of early carotenes in mutant fruit. Carotenoid biosynthetic genes downstream ζ-carotene desaturation in the pathway were also highly expressed in the pulp of Cara Cara fruits indicating a positive feedback regulatory mechanism of carotenoid biosynthesis. Alteration of the carotenoid composition in mutant tissues resulted in lower ABA content but higher *NCED* gene expression, indicating a feedback regulation of ABA biosynthesis in citrus fruits.

4. Experimental

4.1. Plant material, color index and fruit size

Fruits of Navel and Cara Cara oranges (*C. sinensis* L. Osbeck) at ten different developmental stages, from immature-green to full-ripened, were harvested at random from 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 (2004/2005 and 2005/2006). Fruit color 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 color was expressed as the *a/b* Hunter ratio, a classical relationship for color measurement in citrus fruits (Stewart and Wheaton, 1972). The data of fruit diameter and color index for each developmental stage are the means ± SD of 30 replicate samples.

Flavedo (the outer colored 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. All results presented are representative data from one season.

4.2. 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). 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 twice.

4.3. HPLC analysis of carotenoids

Carotenoid composition of each sample was analyzed 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). A C30 carotenoid column (250 × 4.6 mm, 5 μm) coupled to a C30 guard column (20 × 4.0 mm, 5 μm) (YMC Europe GMBH, Germany) was used. Samples were prepared for HPLC by dissolving the dried carotenoid

extracts in $CHCl_3$: MeOH: acetone (3:2:1, v:v:v). A ternary gradient elution was used for carotenoid separation. The initial solvent composition consisted of 90% MeOH, 5% water and 5% methyl *tert*-butyl ether (MTBE). The solvent composition changed in a linear fashion to 95% MeOH and 5% MTBE at 12 min. During the next 8 min the solvent composition was changed to 86% MeOH and 14% MTBE. After reaching this concentration the solvent was gradually changed to 75% MeOH and 25% MTBE at 30 min. Following 20 min solvent composition changed linearly being, at 50 min, 50% MeOH and 50% MTBE. The final composition was reached at 70 min and consisted of 25% MeOH and 75% MTBE. The initial conditions were re-established in 5 min and equilibrated for 15 min before the next injection. The flow rate was 1 ml min^{-1}, column temperature was set to 25 °C and the injection volume was 20 μl. The photodiode array detector was set to scan from 250 to 540 nm, and for each elution a Maxplot chromatogram was obtained, which plots each carotenoid peak at its corresponding maximum absorbance wavelength.

Carotenoids were identified by their retention time, absorption and fine spectra (Rouseff et al., 1996; Britton, 1998). The carotenoid peaks were integrated at their individual maxima wavelength and their content were calculated using calibration curves of β-apo-8′-carotenal (a gift from Hoffman-LaRoche) for β-citraurin, β-carotene (Sigma) for α- and β-carotene, β-cryptoxanthin (Extrasynthese) for α-and-β-cryptoxanthin, lutein (Sigma) for lutein, neoxanthin and violaxanthin isomers, lycopene (Sigma) for lycopene, and zeaxanthin (Extrasynthese) for zeaxanthin. Standards of phytoene and phytofluene for quantification were obtained from flavedo extracts of Pinalate fruits, which accumulate large amounts of these compounds (Rodrigo et al., 2003), and afterward purified by TLC (Pascual et al., 1993).

4.4. Probe synthesis and labeling

Probes were derived from cDNA clones of the carotenoid biosynthetic genes *PSY*, *PDS*, *ZDS*, *PTOX*, β-*LCY*, ε-*LCY*, β-*CHX*, *ZEP* (Rodrigo et al., 2004), ABA biosynthetic genes *NCED1* and *NCED2* (Rodrigo et al., 2006) and 26rDNA (Ballester et al., 2006) from *C. sinensis*.

A *C. sinensis* sequence (GenBank CF504139) with high homology (77% identity at amino acid level) to *Arabidopsis thaliana* ε-*CHX* (*LUT1*) gene was obtained from public databases. Based on that sequence, a pair of primers, MJ89 (sense) 5′-CGGCACCAAGTATGCTAAAGG-3′ and MJ90 (antisense) 5′-CAGCAGTTCCATTTAGAGGG-3′, were designed. A partial cDNA clone 221 bp long corresponding to *C. sinensis* ε-*CHX* gene was obtained by RT-PCR using cDNA from flavedo tissue of Navel fruits at the IG stage. The cDNA synthesis reaction was carried out in the presence of 500 ng of oligo-dT with 200 units of Superscript II Reverse Transcriptase (Gibco BRL, Karlsruhe, Germany). An EST sequence from *C. sinensis* of 246 bp, identical to GenBank CX048486, with 77% of identity at amino acid level to *Catharanthus roseus* DXS (GenBank AJ011840) was used as template for *DXS* probe-labeling. A *C. clementina* cDNA clone of 191 bp (GenBank CX293560) with 94% amino acid identity with *Solanum lycopersicon* HDS was used as template for *HDS*-probe labeling. A *C. clementina* cDNA clone of 463 bp (GenBank CX298449), which presented 84% amino acid identity with *Arabidopsis* HDR (GenBank AAW82381) was used as template for *HDR*-probe labeling.

All probes were labeled with [α-^{32}P]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}) was used for each hybridization.

4.5. 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 were performed as described previously (Rodrigo et al., 2004). 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 Navel flavedo at full-colored stage and expression level of the rest of the samples referred to it.

4.6. RT-PCR analysis

Total RNA was treated with DNase (Ambion, Huntingdon, UK) and accurately quantified by fluorometric assay with the RiboGreen dye (Molecular Probes) following the manufacturer's instructions, in order to normalize mRNA levels as described by Alos et al. (2006). Quantitative real-time was performed with a LightCycler 2.0 Instrument (Roche) and fluorescence was analysed using LightCycler Software version 4.0. One-step RT-PCR was carried out on 100 ng total RNA adding 2.5 units of MultiScribe Reverse Transcriptase (Applied Biosystems), 1 unit Rnase Inhibitor (Applied Biosystems), 2 μl LC FastStart DNA MasterPLUS SYBR Green I (Roche) and 0.5 μM of gene specific primers in a total volume of 10 μl. For *DXS*, the primers and the RT-PCR procedure used were that described by Alos et al. (2006). Primers pairs for β-*LCY* [MJ136 (sense) 5′-GAACCAGGAGCTTAGGTCTG-3′ and MJ137 (antisense) 5′- GCTAGGTCTACAACAAGGCC-3′] and *HDS* [MJ154 (sense) 5′-CTGCCGGAAATTGGACTTCC-3′ and MJ155 (antisense) 5′-CCATCCTGAAGAAGGGTACC-3′] were designed based on *Citrus* coding sequences isolated from fruit and available in databases (GenBank DQ232259 and GenBank CX293560, respectively). The RT-PCR procedure consisted of 48 °C 30 min, 95 °C 10 min followed by 35 cycles at 95 °C 10 s, 55 °C 5 s and 72 °C 10 s. Fluorescence intensity data were acquired during the 72 °C extension step and specificity of the reactions was checked by post-amplification dissociation curves. To transform fluorescence intensity measurements into relative mRNA levels, a 10-fold dilution series of a RNA sample was used as a standard curve. Values were the mean of at least three independent analyses. An expression value of 1 was arbitrary assigned to the IG sample.

4.7. ABA analysis

Plant material used for ABA quantification was the same as that used for pigment analysis. Quantification of ABA in flavedo and pulp of Navel and Cara Cara fruits was performed by indirect enzyme-linked immunosorbent assay as reported previously (Zacarias et al., 1995; Lafuente et al., 1997).

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