

Exploring the diversity in *Citrus* fruit colouration to decipher the relationship between plastid ultrastructure and carotenoid composition

Joanna Lado^{1,2} · Lorenzo Zacarías¹ · Aranzazu Gurrea¹ · Anton Page³ · Anthony Stead⁴ · María J. Rodrigo¹

Received: 13 March 2015 / Accepted: 9 July 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract

Main conclusion Differentiation of new and characteristic plastid ultrastructures during ripening of citrus fruits in both peel and pulp appears to be strongly correlated with the content and complement of carotenoids.

Most of the species of the *Citrus* genus display a wide range in fruit colouration due to differences in carotenoids; however, how this diversity is related and may contribute to plastid differentiation and ultrastructure is currently unknown. To that end, carotenoid profile and plastid ultrastructure were compared in peel and pulp of three sweet oranges: the ordinary orange-coloured Navel, rich in β,β -xanthophylls, the yellow Pinalate mutant with an elevated content of colourless carotenes and reduced β,β -xanthophylls, and the red-fleshed Cara Cara with high concentration of colourless carotenes and lycopene in the

pulp; and two grapefruits: the white Marsh, with low carotenoid content, and the red Star Ruby, accumulating upstream carotenes and lycopene. The most remarkable differences in plastid ultrastructure among varieties were detected in the pulp at full colour, coinciding with major differences in carotenoid composition. Accumulation of lycopene in Cara Cara and Star Ruby pulp was associated with the presence of needle-like crystals in the plastids, while high content of upstream carotenes in Pinalate pulp was related to the development of a novel plastid type with numerous even and round vesicles. The presence of plastoglobuli was linked to phytoene and xanthophyll accumulation, suggesting these structures as the main sites for the accumulation of these pigments. Peel chromoplasts were richer in membranes compared to pulp chromoplasts, reflecting their different biogenesis. In summary, differences in carotenoid composition and accumulation of unusual carotenoids are mirrored by the development of diverse and novel chromoplast types, revealing the plasticity of these organelles to rearrange carotenoids inside different structures to allow massive accumulation and thus contributing to the chemical stability of the carotenoids.

Electronic supplementary material The online version of this article (doi:10.1007/s00425-015-2370-9) contains supplementary material, which is available to authorized users.

✉ María J. Rodrigo
mjrodrigo@iata.csic.es

- ¹ Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Avenida Agustín Escardino 7, 46980 Paterna, Valencia, Spain
- ² Instituto Nacional de Investigación Agropecuaria (INIA), Camino a la Represa s/n, Salto, Uruguay
- ³ Biomedical Imaging Unit, Faculty of Medicine, Southampton General Hospital, University of Southampton, Tremona Road, Southampton, UK
- ⁴ School of Biological Sciences, Royal Holloway, University of London, Egham Hill, UK

Keywords Carotenoids · Chloroplasts · Chromoplasts · Citrus fruit · Ripening

Abbreviations

C, Cara	Cara orange
FW	Fresh weight
MSH	Marsh grapefruit
N	Navel orange
P	Pinalate orange
SR	Star Ruby grapefruit
TEM	Transmission electron microscopy

Introduction

Citrus fruit is the most important tree-crop in the world in terms of annual production and colour is one of the most important attributes of fruit quality. Carotenoids are the main pigments responsible for the colour singularities of *Citrus* fruit, and its development during ripening involves coordinated changes in chlorophyll degradation and biosynthesis and accumulation of carotenoids in both peel and pulp tissues. Carotenoids, in addition to the relevant functions in plants (Britton 2008), are also important nutritional components of the human diet, since some of them are precursors of vitamin A and possess beneficial antioxidant capacities, playing then a role in the protection against degenerative and chronic diseases (Berman et al. 2014). Due to these relevant functional properties and their importance in the organoleptic quality of citrus fruit, carotenoid content and composition and their metabolic regulation in the peel and pulp of different citrus species and varieties have been extensively studied over decades (reviewed in Kato 2012; Rodrigo et al. 2013).

Carotenoids are C_{40} isoprenoid lipid-soluble compounds and can be divided into carotenes, which are hydrocarbons, and their oxygenated derivatives, xanthophylls. Phytoene, phytofluene, ζ -carotene and lycopene are examples of linear carotenes, while cyclization in β -ring of both ends of lycopene results in the formation β -carotene and cyclization in β -ring and ϵ -ring in α -carotene. Further modifications on the β - and/or ϵ -rings by adding hydroxyl-, epoxy-, carboxy, keto or ester functional groups derive in the formation of xanthophylls such as lutein, zeaxanthin, β -cryptoxanthin or violaxanthin. Carotenoids are synthesized in the plastids, the most common being the chloro- and chromoplasts, organelles that in addition to their role in essential metabolic pathways, also contribute to the storage of these pigments in specialized structures (Ljubesic et al. 1991; Li and Yuan 2013; Nogueira et al. 2013). Carotenoids in chloroplasts are mainly located in the photosynthetic membranes in the form of chlorophyll–carotenoid–protein complexes involved in light harvesting and photoprotection (Vishnevsky et al. 1999). Chromoplasts are defined as plastids which are photosynthetically inactive (Gross 1987) but have developed unique mechanisms to synthesize and accumulate large amounts of carotenoids. Carotenoids content appears to be determined not only by the rate of biosynthesis and degradation, but also by the capacity of developing stable storage sink structures within the plastids (Li and Yuan 2013). During ripening of citrus fruit peel, the transition from chloroplasts into chromoplasts involves changes in structure, morphology and composition. These changes implicate a controlled breakdown of chlorophyll, a decrease in chloroplastic-type carotenoid content, thylakoid

membrane disassembly, starch degradation and the appearance of new sites for carotenoid biosynthesis and accumulation, such as plastoglobuli (Gross 1987). Plastoglobuli are lipid bodies, lacking a surrounding membrane, containing different carotenoid biosynthetic enzymes among other proteins and constitute a structure where carotenoids are synthesized, sequestered or stored (Ytterberg et al. 2006; Br  h  lin et al. 2008; Li and Yuan 2013; Nogueira et al. 2013). Plastoglobuli have been reported from the chromoplasts of numerous different carotenoid-containing fruits (Vasquez-Caicedo et al. 2006; Schweigert et al. 2011; Nogueira et al. 2013), including citrus (Thomson 1966; Burns et al. 1992; Zeng et al. 2011; Cao et al. 2012), and increase in size and number during ripening (Ljubesic et al. 1991). Other structures have been described to coexist with plastoglobuli in the chromoplasts, and the relative abundance of each type of structure leads to the classification as globular, tubular, membranous and crystalloid chromoplasts (Sitte et al. 1980; Egea et al. 2010). Moreover, the development of sub-chromoplast structures seems to be related to the type of carotenoids that are sequestered (Ljubesic et al. 1991; Nogueira et al. 2013), and these structures may act also as a physical barrier influencing the bioaccessibility of carotenoids from fruits and vegetables (Jeffery et al. 2012).

Citrus fruits are one of most complex sources of carotenoids, with a large diversity of these compounds among the different species and cultivars in terms of types and amounts (Kato et al. 2004; Fanciullino et al. 2006; Xu et al. 2006; Kato 2012; Rodrigo et al. 2013). Interestingly, a common feature in mature citrus fruit is that carotenoid concentrations are usually higher in the peel than in the flesh (juice vesicle), and most of the citrus species and varieties display a different carotenoid profile in these tissues, suggesting differential regulation of biosynthesis and accumulation of these pigments in these fruit tissues (Tadeo et al. 2008). Early electron microscopy studies of chromoplasts in citrus fruit revealed the presence of electron-dense plastoglobuli (400–800 nm) combined with membranes in mature Valencia oranges (Thomson 1966). By contrast, two types of chromoplasts were observed in flavedo (outer coloured layer of fruit peel) cells of ripe pummelo (*Citrus maxima*), one of the citrus ancestral species: a typical one containing large plastoglobuli and vesicles, and an unusual and more abundant type containing plastoglobuli but also long concentric achlorophyllous membranes (Gross et al. 1983). Plastoglobuli were also reported to be present in flavedo chromoplasts of Satsuma mandarin (Shimokawa et al. 1978), grapefruit (Gross 1987) and lemon (Ljubesic 1984). The chromoplasts from the pulp of the red Star Ruby grapefruit were described as sparse and exclusive to the outer cells of the juice sacs and

seemed to be dominated by electron-transparent vesicles and electron-dense background material (Jeffery et al. 2012). Cao et al. (2012) have classified the chromoplasts of the peel of Marsh grapefruit, Cara Cara orange and Sunburst mandarin as globular, due to the presence of large numbers of plastoglobuli.

The relationship between the type of sub-chromoplast structures and the carotenoid content and composition during ripening of citrus fruit is not well understood and whether the accumulation of specific carotenoids may be a key factor determining the differentiation and development of particular carotenoid-containing structures in the chromoplast is currently unknown. Therefore, taking advantage of the great variability in fruit carotenoid composition among sweet oranges and grapefruits, the main objective of the present study has been to investigate the changes in chromoplast ultrastructure during ripening in relation to the carotenoid complement in fruits of selected varieties from these citrus species. To that end, fruits from the ordinary Navel sweet orange (*Citrus sinensis* L. Osbeck), with the typical standard orange colouration, which accumulates β,β -xanthophylls, mainly 9-(Z)-violaxanthin, as well as lower amounts of colourless carotenoids and apocarotenoids C₃₀ (Oberholster et al. 2001; Rodrigo et al. 2004, 2013), were selected and compared with two sweet orange spontaneous mutants, the yellow Pinalate and the red-pulp Cara Cara. Pinalate is a unique mutant with a distinctive pale-yellow colouration in peel and pulp of mature fruit accumulating significant amounts of early linear carotenes: phytoene, phytofluene and ζ -carotene isomers, while the proportion of β,β -xanthophylls is extremely reduced, implying a blockage at the stage of desaturation of ζ -carotene (Rodrigo et al. 2003). Similarly, a natural mutation derived from the Navel sweet orange has led to the appearance of the red-fleshed Cara Cara (Xu et al. 2006; Alquézar et al. 2008). Cara Cara orange peel contains similar levels of 9-(Z)-violaxanthin to its parental Navel but a higher content of the colourless phytoene, while the flesh displays a reddish colouration due to the presence of lycopene, which is a very uncommon carotene in sweet orange fruit (Xu et al. 2006; Alquézar et al. 2008; Zeng et al. 2011). Among the genus *Citrus*, grapefruit (*Citrus paradisi*) is one of the species with the highest diversity of external and internal fruit colouration, ranging from red or pink to pale yellow (Gmitter 1995). The Marsh white grapefruit is one of the largest cultivated and consumed worldwide and contains only minute amounts of phytoene and violaxanthin in peel and pulp, whereas the red grapefruit Star Ruby is described as one of the varieties with a more intense red colouration (Gmitter 1995), accumulating large amounts of carotenoids, mainly phytoene, phytofluene and lycopene in both tissues (Xu et al. 2006; Cao et al. 2012; Alquézar et al. 2013). Thus, in this

study, we have used the natural variability in two species of citrus fruits to study the relationship between carotenoid content and composition and plastid differentiation.

Materials and methods

Plant material and treatments

Fruit of the ordinary Navel orange (*Citrus sinensis* L. Osbeck) (Saunt 2000), Cara Cara (Lee 2001) and Pinalate (Rodrigo et al. 2003) sweet orange mutants, and the white Marsh and the red Star Ruby grapefruit (*Citrus paradisi* Macf.) (Gmitter 1995) were harvested from adult trees located at the Spanish Citrus Germplasm Bank (IVIA, Moncada, Valencia, Spain) subjected to the same environmental conditions and agronomical practices. For all varieties, fruits were harvested at two ripening stages, breaker (harvested between 230 and 245 days after bloom) and full colour (harvested between 330 and 340 days after bloom), and index colour, size and maturation index of the fruits at harvest are indicated in Table S1. At least 3 replicate samples of 10 fruit each were harvested at each sampling date. Fruits were delivered to the laboratory, selected by size uniformity and those free of any defect were used for pigment analysis or microscopy studies. For pigment analysis, flavedo (external coloured portion of the peel) and pulp tissues were excised and frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C until analysis.

Chlorophyll and carotenoid extraction

Flavedo and flesh pigments were extracted as previously described (Alquézar et al. 2008). The chlorophyll (a+b) content in breaker fruit was determined by measuring the absorbance at 644 and 662 nm and calculated according to the Smith and Benitez equations (Smith and Benítez 1955). After chlorophyll measurements, the pigment ethereal solution was dried and saponified using a 6 % methanolic: KOH solution. Carotenoids were extracted and samples dried under N₂ and kept at -20 °C until analysis. All procedures were carried out on ice under dim light to prevent possible photodegradation, isomerisation and structural changes of carotenoids.

Carotenoid analysis by HPLC

Prior to HPLC analysis, carotenoid extracts were dissolved in acetone and incubated overnight at -20 °C to precipitate sterols which could interfere in the carotenoid analysis and subsequently dried under N₂. Carotenoid composition of each sample was analysed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a

model 2998 photodiode array detector, and Empower software (Waters). 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) was used. Samples were prepared for HPLC by dissolving carotenoid extracts in CHCl₃: MeOH: acetone (3:2:1, by vol.). A ternary gradient elution with MeOH, water and methyl *tert*-butyl ether (MTBE) was used for carotenoid separation (Alquézar et al. 2008). Carotenoids were identified by comparison of the spectra and retention time with those of authentic standards, when available, or by matching the observed versus literature spectral data and retention time under identical chromatographic conditions (Britton 1995; Rodrigo et al. 2004). The carotenoid peaks were integrated at their individual maxima wavelength, and their contents were calculated using calibration curves of β-cryptoxanthin (Extrasynthese), lutein (Sigma) for lutein and neoxanthin, zeaxanthin (Extrasynthese), α- and β-carotene (Sigma) and lycopene (Sigma). Standards of phytoene, phytofluene and ζ-carotene were obtained from peel extracts of Pinalate orange fruits (Rodrigo et al. 2003), and of all-*E*-violaxanthin and 9-*Z*-violaxanthin from peel extracts of Navel orange fruits (Rodrigo et al. 2004) and HPLC purified. Samples were extracted twice, and each analytical determination was replicated at least twice. Sample preparation was carried out on ice under dim light to prevent photodegradation, isomerisation and structural changes of carotenoids.

Transmission electron microscopy (TEM)

The peel and pulp tissue samples were manually cut in small squares of 5 mm long × 1 mm wide, fixed in modified Karnovsky fixative (0.5 % glutaraldehyde, 2.5 % formaldehyde in 0.1 M phosphate buffer pH 7.4). Specimens were then rinsed in 0.1 M PIPES buffer, postfixed in 1 % buffered osmium tetroxide (1 h), rinsed in buffer, block stained in 2 % aqueous uranyl acetate (20 min), dehydrated in an ethanol series and embedded in Spurr resin (Elektron Technology, Stansted, UK) in the normal way. Gold/silver ultrathin sections were then cut from suitable regions of the tissue and stained with Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope equipped with a SIS Megaview III digital camera. For quantification of the average number of plastids/cell, the four layers of cells below the epidermis were examined.

Polarized light microscopy

For polarized light visualization, fresh pulp vesicles were carefully extracted and placed onto a glass slide, cut through the midline with a scalpel and covered with a glass-coverslip without any fixative. Images were taken with a Nikon Eclipse 90i (Japan) coupled with a polarization filter.

Results

Changes in pigment composition in peel of oranges and grapefruits at two ripening stages

Chlorophyll and carotenoid content were examined in the peel of the ordinary (Navel, N) and two mutants (the yellow Pinalate, P and the red Cara Cara, C) oranges and in white (Marsh, MSH) and red (Star Ruby, SR) grapefruits, at two maturation stages, breaker and full colour. These stages were selected because previous studies have demonstrated that the transition from chloroplast to chromoplast occurs at colour-break, concomitant with massive increase in coloured carotenoids that finally determine the characteristic colouration of full mature fruits (Gross 1987; Kato et al. 2004; Rodrigo et al. 2004; Alquézar et al. 2013).

Peel colour was similar in fruits of the three orange varieties at the breaker stage (Table S1). In full-coloured fruit, P presented the characteristic pale-yellow colouration and N and C were similar to each other showing orange colour. Conversely, grapefruits showed, as expected, marked differences in colouration, since at the same ripening stage, white grapefruit was always less coloured than the red SR (Table S1). Chlorophyll content in the peel of P fruit (154 μg g⁻¹ FW) at the breaker stage was around 30 % higher than in N and C (106 and 100 μg g⁻¹ FW, respectively), in agreement with the slow rate of peel colouration in this mutant (Rodrigo et al. 2003). In the peel of grapefruit at breaker stage, chlorophylls were 30 % higher in SR (179 μg g⁻¹ FW) than in MSH (125 μg g⁻¹ FW). In the peel of full-coloured fruit of all varieties, only residual amounts (below 5.0 μg g⁻¹ FW) of chlorophylls were detected. Analysis of carotenoid profile by HPLC-PDA revealed important qualitative and quantitative differences in the peel of the five varieties. At breaker stage, the peel of P accumulated 3 times (52.4 μg g⁻¹ FW) more carotenoids than the parental N (16.3 μg g⁻¹ FW) and also the red mutant C (13.9 μg g⁻¹ FW) (Fig. 1a). In the peel of mature fruit, total carotenoids increased 7.5-, 9- and 13-fold in P, N and C fruits, respectively, compared to values at breaker stage, and the differences between varieties were also maintained (Fig. 1c). Total carotenoid concentration in the peel of MSH grapefruit (8.0 μg g⁻¹ FW) was relatively low and similar at breaker and full colour stages; by contrast, SR peel accumulated larger amounts of carotenoids that moderately increased from breaker (41.6 μg g⁻¹ FW) to full-coloured fruit (55.4 μg g⁻¹ FW) (Fig. 1b, d).

As expected, there were large differences in fruit peel carotenoid composition among the varieties studied. The peel of P fruit accumulated substantial amounts of linear carotenoids, mainly phytoene (28.6 μg g⁻¹ FW), phytofluene (4.69 μg g⁻¹ FW) and ζ-carotene (4.62 μg g⁻¹

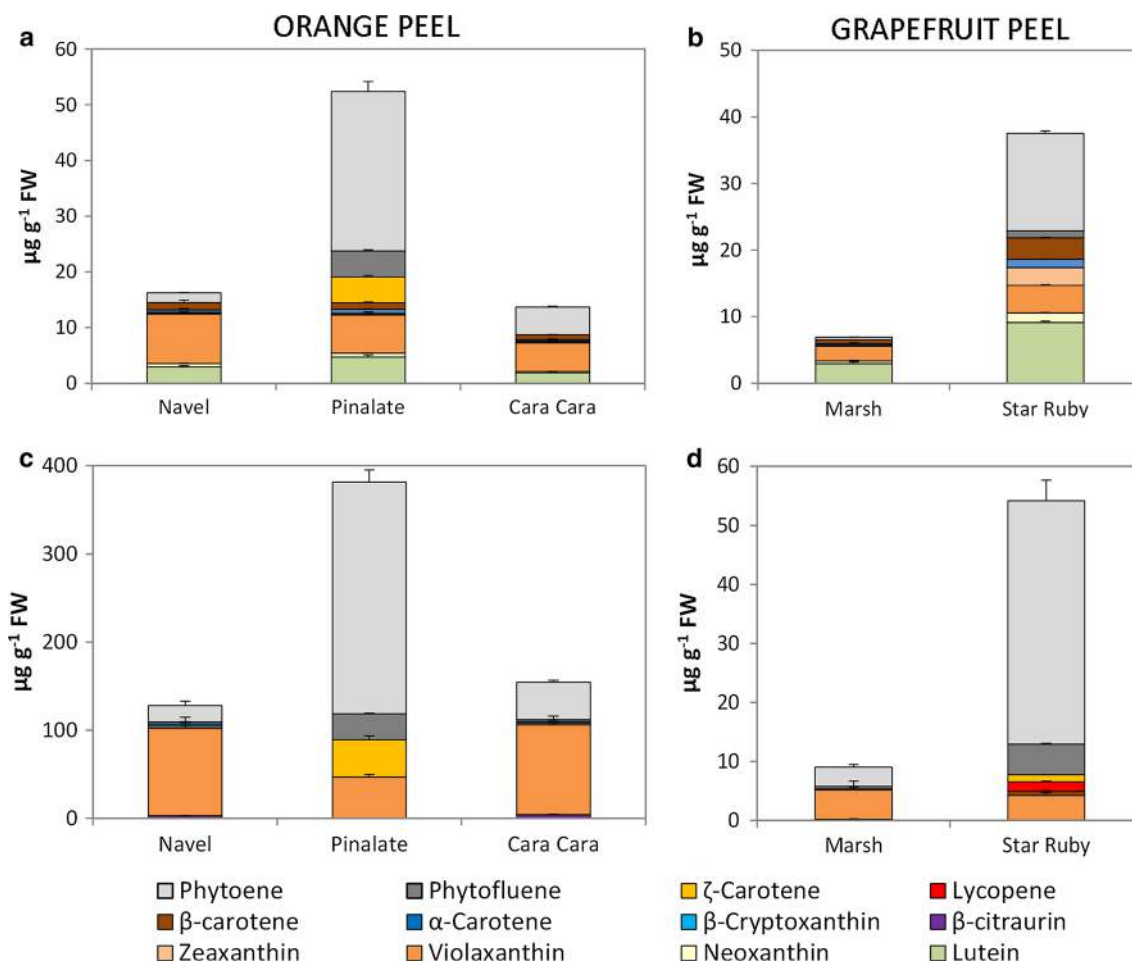


Fig. 1 Carotenoid content and composition ($\mu\text{g g}^{-1}$ FW) in the peel of fruit of three oranges (*Citrus sinensis* L) (a, c), the ordinary orange-coloured N, and the yellow P and the red C mutants, and two

grapefruit (*Citrus paradisi*) (b, d), the white MSH and the red SR, at breaker (a, b) and full colour (c, d) stages. Data of carotenoid contents are mean \pm SE of three replicates

grapefruit (Fig. 1a). By contrast, phytoene represented only 10 % of total carotenoids in peel of N fruits and 36 % in the C mutant. At this breaker stage, other chloroplastic-type carotenoids (i.e. lutein, β - and α -carotene) were also detected in the peel of all orange varieties (Fig. 1a). The increase in total carotenoids during peel colouration was accompanied by a change in their profile (Fig. 1c). Thus, the peel of P mature fruit showed an extraordinary increment in the content of linear carotenoids, mainly phytoene ($263 \mu\text{g g}^{-1}$ FW) but also in phytofluene ($29.5 \mu\text{g g}^{-1}$ FW) and an important amount of a mix of ζ -carotene isomers ($42.2 \mu\text{g g}^{-1}$ FW), all accounting for 86 % of total carotenoids, and the remaining 14 % corresponded almost exclusively to 9-(Z)-violaxanthin (Fig. 1c). By contrast, the xanthophyll 9-(Z)-violaxanthin was predominant in the peel of N and C, accounting for 68 and 57 % of total carotenoids, respectively (Fig. 1c). The C_{30} apocarotenoid β -citraurin accumulated progressively with peel colouration of N ($2.76 \mu\text{g g}^{-1}$ FW) and C ($4.15 \mu\text{g g}^{-1}$ FW)

oranges but it was not detected in the yellow P mutant (Fig. 1c).

Total carotenoids in the peel of SR grapefruit at both ripening stages were 4–5 times higher than in MSH. In both grapefruits at the breaker stage, chloroplastic-type carotenoids represented around 30 % of the total content (Fig. 1b). In SR, linear carotenoids represented about 40 and 89 % of total carotenoids in breaker and full-coloured fruit, respectively, with the striking feature of the accumulation of the red carotene lycopene ($1.6 \mu\text{g g}^{-1}$ FW) at the full-coloured stage. By contrast, the peel of mature MSH grapefruit only accumulated minor amounts of carotenoids, mainly phytoene ($3.3 \mu\text{g g}^{-1}$ FW) and violaxanthin ($4.98 \mu\text{g g}^{-1}$ FW; Fig. 1d).

Plastid ultrastructure in the peel of oranges and grapefruits at two ripening stages

Thin cut sections of flavedo tissue of the three orange and two grapefruit varieties at breaker and full-colour stage

were obtained, and the first 4–5 layers of cells underneath the epidermis (Suppl. Fig. S1) were examined by transmission electron microscopy (TEM) for the presence of chloro- and chromoplasts. In the peel of breaker oranges, chloroplasts were observed surrounding the vacuole of epidermal and outer exocarp cells. Inside the chloroplast, grana composed of stacked thylakoids were detected as well as starch grains. At this stage, most chloroplasts showed an advanced disintegration of thylakoid membranes concomitant with the accumulation of smooth round osmiophilic plastoglobuli (Fig. 2a–f). These particular chloroplastic structures were detected in the epidermis and cells of the flavedo of the three oranges varieties, and the number of chloroplasts was also comparable with an average of 4.5 ± 0.5 per cell. Similar to oranges, no obvious differences were detected in the chloroplast structure of both grapefruit varieties at breaker stage, showing similar plastid number per cell as well as a combination of intact thylakoid membranes with membranes progressively disassembled (Fig. 3a–d). In grapefruit flavedo plastids, plastoglobuli were also present while only small starch grain remnants were detected (Fig. 3a, b, d).

As peel colouration progressed, chloroplasts disappeared and were transformed into chromoplasts. The chromoplasts number was reduced to an average of 2.3 ± 0.2 per cell in the epidermis and exocarp of N and C, whereas in the orange P mutant, the reduction in the number of chromoplasts was less marked (3.0 ± 0.3 per cell). Moreover, in P mutant, massive amounts of starch grains were easily distinguishable in thick sections examined by light microscopy after toluidine blue staining, while in ordinary N orange and the red C mutant, only low amounts of starch grains could be observed (Suppl. Fig. S1). Besides these particular features (higher plastid number per cell and starch grains) in the peel of P mutant, it was remarkable the presence of massive uneven storage lipid droplets (LD) (300–800 nm in diameter) inside the plastids, being less electron-dense than plastoglobuli. These structures appeared to be extruded from the chromoplasts when reaching a particular size or number, and then accumulated in the cytosol surrounded by membranes (Fig. 2i, Suppl. Fig. S3). This phenomenon was rarely observed in C and N chromoplast, where released lipid structures formed massive drop-like complexes in the cytosol (Suppl. Figs. S4 and S5). Typical even, round and highly osmiophilic plastoglobuli were present in both N and C chromoplasts, being of smaller diameter in N (180–370 nm) than in C (180–550 nm) (Fig. 2g, k), while barely or not detectable in P peel (Fig. 2i, j). In the peel of the three orange varieties, achlorophyllous membranous structures inside peel chromoplasts were detected, forming parallel organized structures (Fig. 2h, j, l).

Chromoplasts from the flavedo of coloured yellow MSH grapefruit contained typical round smooth plastoglobuli (50–500 nm in diameter), combined with membranous structures (Fig. 3e, f). In the red SR grapefruit, the chromoplasts contained irregular round and less electron-dense droplets (Ird), round highly osmiophilic plastoglobuli (Pg) and arranged membranous structures (mm) (Fig. 3g, h). The average number of chromoplasts per cell was slightly higher in SR (3.0 ± 0.3) than in MSH (2.0 ± 0.2).

Changes in pigment composition in pulp of oranges and grapefruits at two ripening stages

Carotenoid content and composition were also analysed in pulp of the orange and grapefruit varieties (Fig. 4) selected at the same ripening stages used for pigment analysis in the peel. The pulp of breaker fruit of N and P oranges was yellowish and similar to each other, but in the C mutant, it already displayed a pale red colouration (Table S1, Fig. 5). Differences in pulp colouration were more evident among varieties in mature fruits, where pulp of P showed a characteristic pale-yellowish colouration, N with a slight orange colour and the pulp of C displayed a red tone (Table S1, Fig. 5). In grapefruits, the pulp of MSH exhibited the typical pale-yellow colouration, and although intensity increased during ripening, it remained yellow in mature fruits. By contrast, the red colour of the SR pulp was evident from the breaker stage and increased with ripening (Table S1, Fig. 6).

Changes in carotenoid content and composition in the pulp of the orange and grapefruit varieties (Fig. 4) and chlorophyll were not detected at any developmental stage in the pulp. Total carotenoids in the pulp of oranges increased with maturity and were approximately 6-times higher in both mutants compared to the ordinary N orange (Fig. 4a, c). Carotenoid composition was also completely different in the pulp of the two mutants, reflecting their differences in colouration. In the pulp of the ordinary N orange, β, β -xanthophylls predominated, mainly the 9-(Z) isomer of violaxanthin, which accounted for 55 and 78 % of total carotenoids in breaker and full colour stages, respectively. At this later stage, phytoene ($1.10 \mu\text{g g}^{-1}$ FW), phytofluene ($0.19 \mu\text{g g}^{-1}$ FW) and the xanthophylls β -cryptoxanthin ($0.59 \mu\text{g g}^{-1}$ FW) and zeaxanthin ($0.21 \mu\text{g g}^{-1}$ FW) were also detected (Fig. 4c). In the pulp of both P and C mutants, the most striking feature was the extremely high amount of linear carotenes which represented 80–95 % of total carotenoids. In P and C pulp, 15-(Z)-phytoene and phytofluene accounted for 50–80 % of the total content, while in P pulp, ζ -carotene (mix of isomers) represented between 15 and 19 % of total carotenoids, and in C pulp, lycopene ranged between 20 and 14 % of the total content of

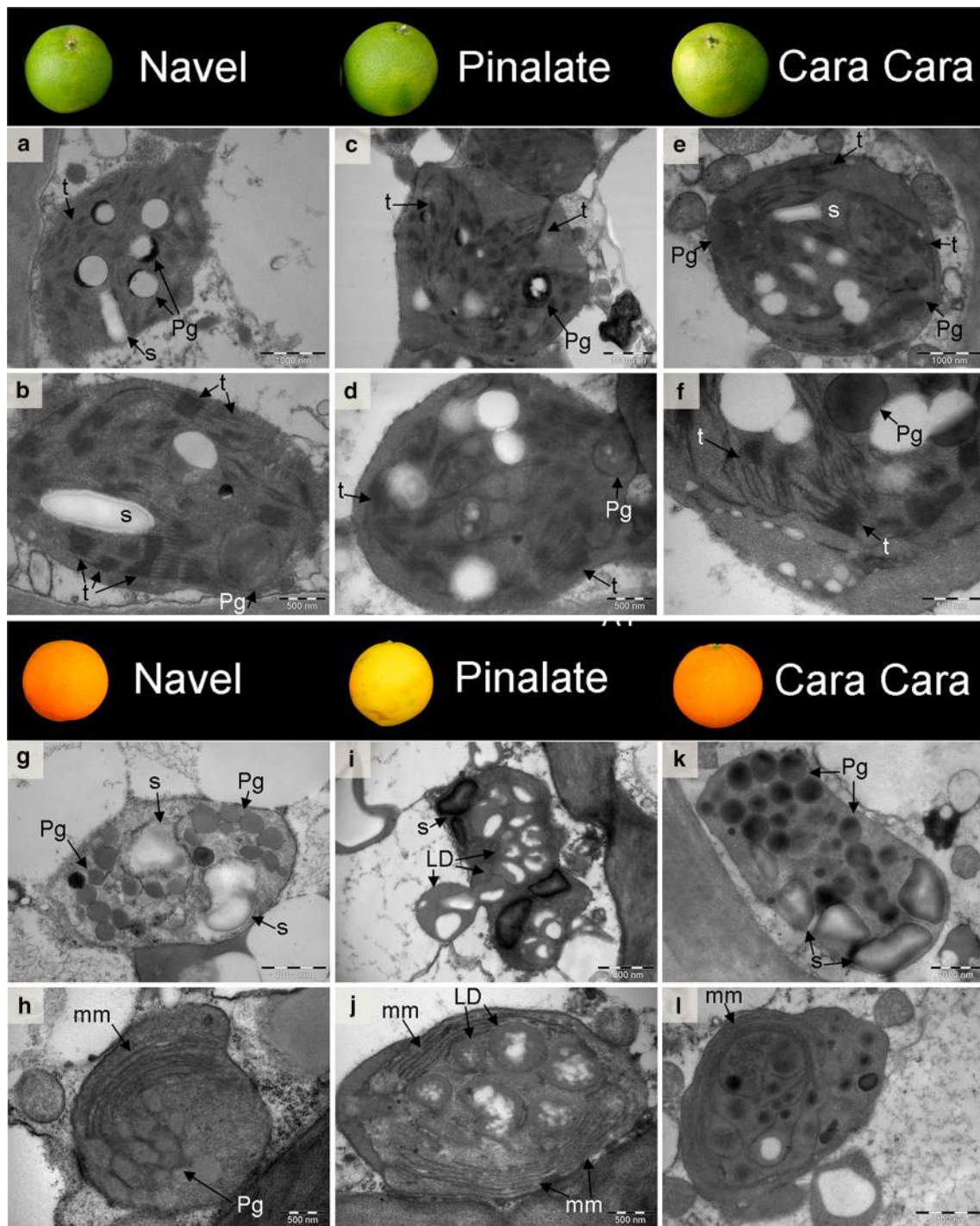


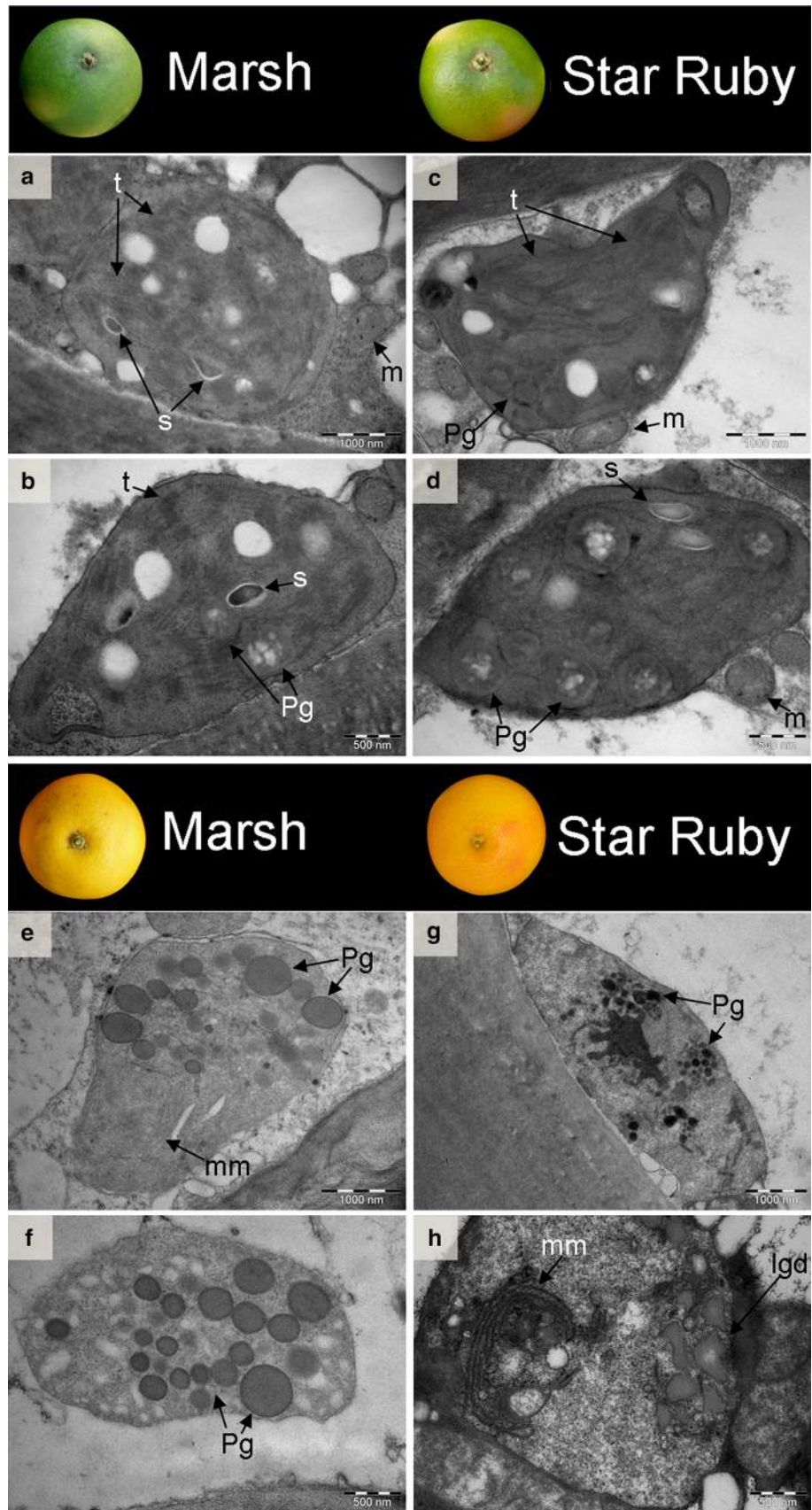
Fig. 2 TEM images showing the chloro- and chromoplasts ultrastructure of the peel of orange fruit (N, P, C) at breaker (a–f) and full colour (g–l) stages. LD lipid droplets, m mitochondria, mm achlorophyllous membranes, n nucleus, Pg plastoglobuli, s starch grains, t thylakoids

carotenoids. In the pulp of P, the content of β,β -xanthophylls was very low, even at full-coloured stage ($2.8 \mu\text{g g}^{-1}$ FW), representing only 4 % of total carotenoids (Fig. 4c), while in C pulp, the concentration of β,β -xanthophylls, mainly 9-(Z) isomer of violaxanthin, was similar to the parental N, but due to the high

concentration of carotenes, its overall percentage contribution was reduced (6.6 %) (Fig. 4c).

Total carotenoid content in the pulp of the red SR was about 90- and 40-times higher than in the white MSH at breaker and full colour stages, respectively. This latter variety accumulated very low amounts of phytoene

Fig. 3 TEM images showing the chloro- and chromoplasts ultrastructure of the peel of grapefruits (MSH and SR) at breaker (a–d) and full colour (e–h) stages. *Igd* irregular low electron-dense droplet, *m* mitochondria, *mm* achlorophyllous membranes, *Pg* plastoglobuli, *s* starch grains, *t* thylakoids



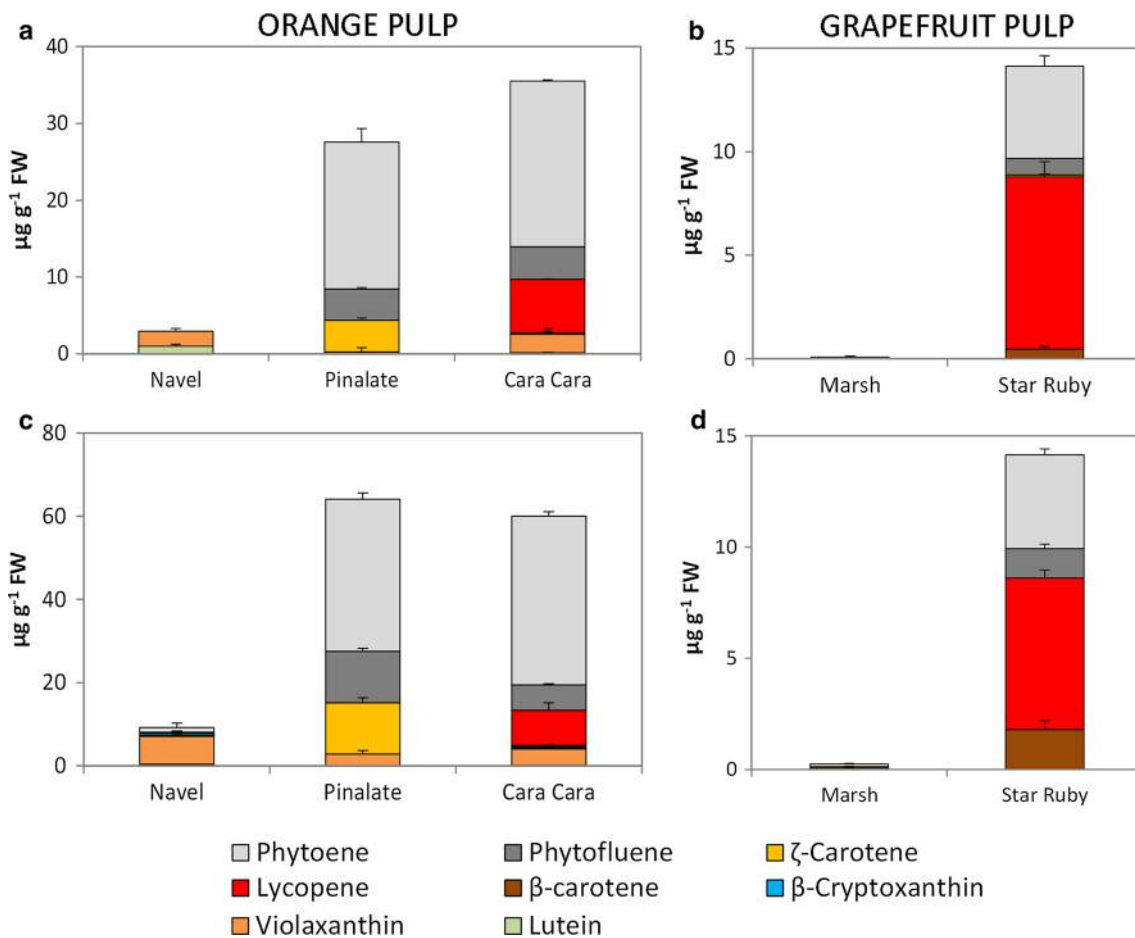


Fig. 4 Carotenoid content and composition ($\mu\text{g g}^{-1}$ FW) in the pulp of fruits of three orange (*Citrus sinensis* L.) (a, c), the ordinary orange-coloured N, and the yellow P and the red C mutants, and two

grapefruit (*Citrus paradisi*) (b, d), the white MSH and the red SR, at breaker (a, b) and full colour (c, d) stages. Data of carotenoid contents are mean \pm SE of three replicates

($0.12 \mu\text{g g}^{-1}$ FW), ζ -carotene ($0.06 \mu\text{g g}^{-1}$ FW) and violaxanthin ($0.07 \mu\text{g g}^{-1}$ FW) at full colouration. By contrast, SR accumulated linear carotenoids ($12.3 \mu\text{g g}^{-1}$ FW), with a major proportion represented by lycopene and phytoene (50 and 30 % of total carotenoids, respectively) and minor amounts of phytofluene and also the cyclic β -carotene ($1.80 \mu\text{g g}^{-1}$ FW) (Fig. 4b, d).

Plastid ultrastructure in the pulp of oranges and grapefruits at two ripening stages

The observation of chromoplast ultrastructure in cells of the pulp (juice vesicles) from the three oranges (Fig. 5) and the two grapefruit varieties was also performed, as striking differences in carotenoid content and composition were detected between them. Thin sections from vesicle stalks were prepared from individual juice vesicles, and cells were inspected for plastid morphology and ultrastructure (cf Suppl. Fig. S2). At breaker stage of all varieties analysed, no chloroplasts were observed in the pulp cells;

however, chromoplasts containing plastoglobuli, membranes and starch grains were detected (Fig. 5a–f). Starch grains were only identified at breaker stage in the pulp of the yellow P and the red C, but not in chromoplasts of the parental N (Fig. 5a–f). By contrast, membranous structures were observed inside chromoplasts of N oranges (Fig. 5a, b) but not in the plastids of either C or P mutants. The abundance of plastoglobuli inside plastids in the pulp of coloured orange fruit increased; the ordinary N orange presented a characteristic type of chromoplast (globular-membranous type), containing typical round smooth and highly osmiophilic plastoglobuli of 100–300 nm in diameter (Fig. 5g, h). Interestingly, in the pulp of the yellow P mutant, which accumulated very high amounts of colourless linear carotenoids, two different kinds of chromoplasts were observed. The chromoplast-type I was quite similar to that of N pulp with typical plastoglobuli of 50–200 nm in diameter (Fig. 5j). The chromoplast-type II (vesicle-type) of P was the most abundant and contained round, electron-lucent membrane-bound vesicles of 250–800 nm in

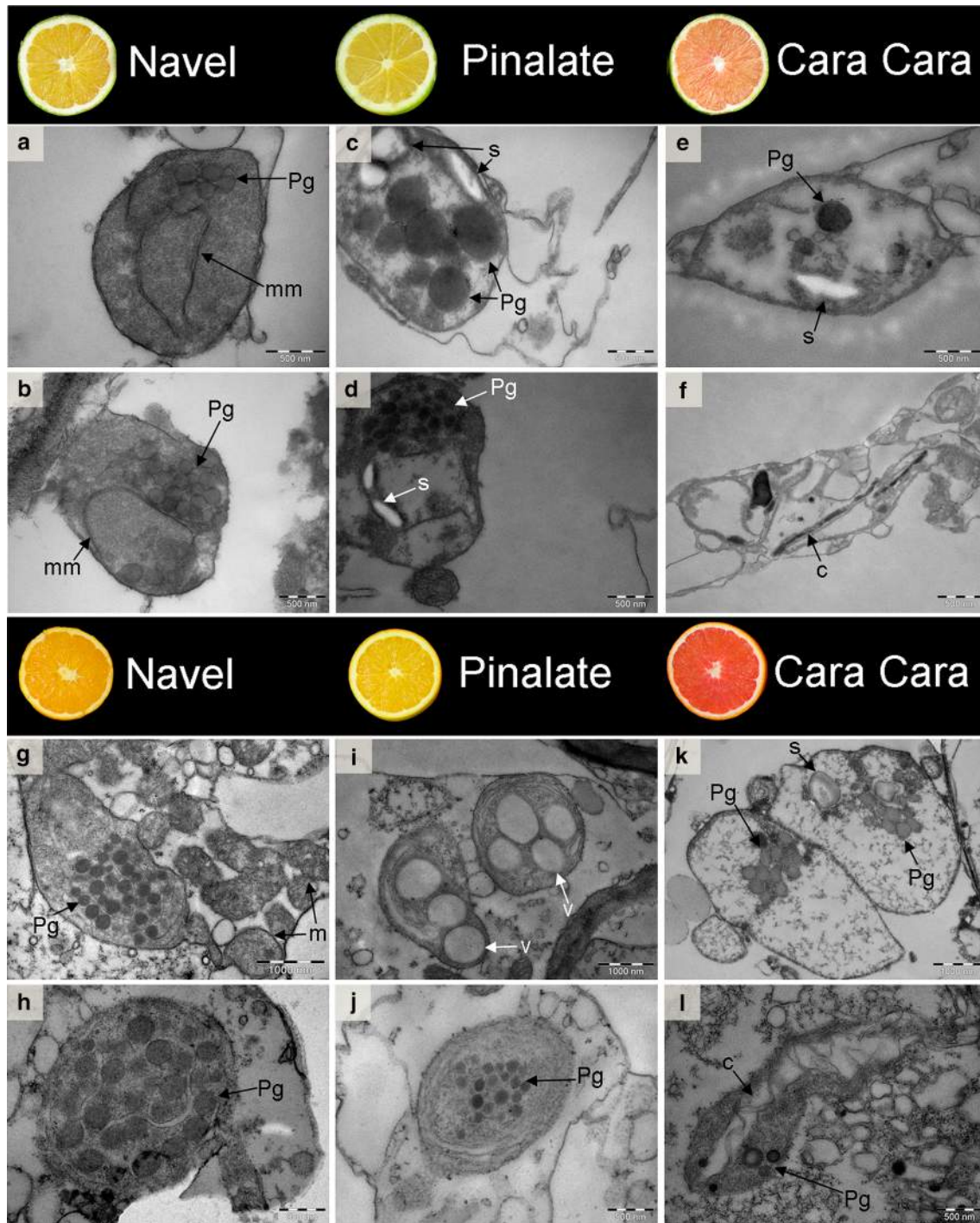


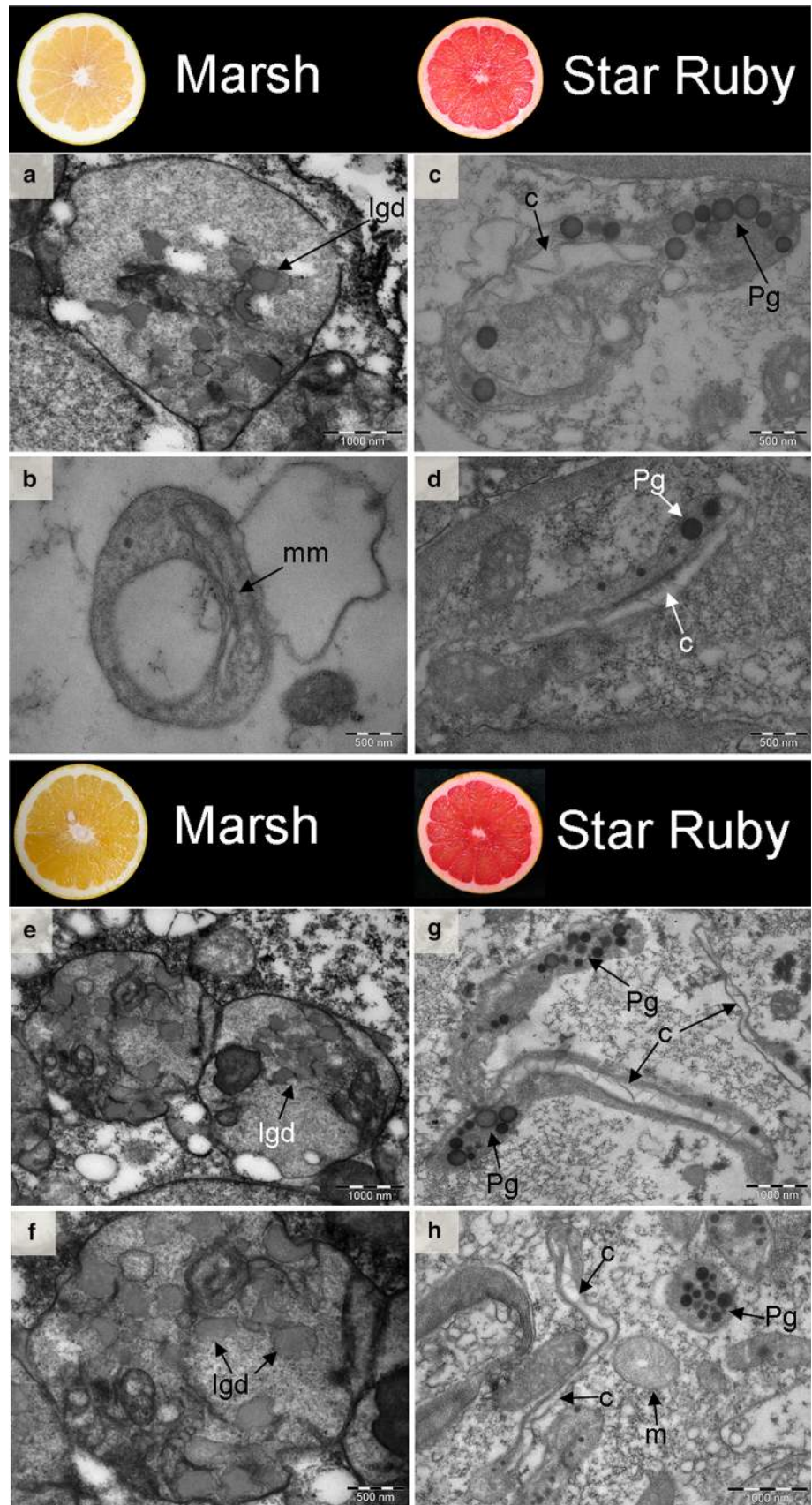
Fig. 5 TEM images showing the chromoplast ultrastructure of the pulp of orange fruit (N, P and C) at breaker (**a–f**) and full colour (**g–l**) stages. *c* lycopene crystals with membrane remnants inside (after osmium fixation the crystalloids are lost during the dehydration

procedure and their expanded envelopes are shrunken into an undulating shape), *m* mitochondria, *mm* achlorophyllous membranes, *Pg* plastoglobuli, *s* starch grains, *v* even and round vesicles only detected in P

diameter (Fig. 5i). Type II and type I chromoplasts were found inside the same cells in a 5:1 ratio. In the pulp of the red C mutant, in addition to the typical globular-membranous chromoplasts containing plastoglobuli of 200–250 nm

in diameter (Fig. 5k), a new type (crystalloid-type) was observed with a novel structure: a long dark structure that resembles lycopene crystals and membranous remnants surrounding them (Fig. 5l). The presence and size

Fig. 6 TEM images showing the chromoplasts ultrastructure of the pulp of grapefruits (MSH and SR) in breaker (a–d) and full colour (e–h) stages. *c* lycopene crystals with membrane remnants inside (after osmium fixation, the crystalloids are largely dissolved during the dehydration procedure, and their expanded envelopes are shrunken into an undulating shape), *Igd* irregular grey droplets, *Pg* plastoglobuli



(2–14 μm long) of crystals in the pulp of C were confirmed by polarized light microscopy (Fig. 7b). These structures were not observed in the pulp of either of the other two oranges (Fig. 7c, d), in agreement with the absence of lycopene in the pulp of these varieties (Fig. 4).

Differences in the structure of chromoplasts from the pulp were even more noticeable in grapefruits, as both varieties also showed dramatic differences in carotenoid content and composition. The red pulp of SR presented a high proportion of crystals in the chromoplasts, which were evident from the breaker stage (Fig. 6c, d) and resembled those present in crystalloid-type C chromoplasts (Fig. 5l). Interestingly, TEM observation suggested the presence of a higher number of crystals in plastids from SR grapefruits than in C oranges, which was further corroborated by polarized light microscopy (Fig. 7a, b). Moreover, the presence of typical round smooth plastoglobuli with or without crystals was also detected in chromoplasts of SR pulp at full colour stage (Fig. 6g, h). In the pulp of the white MSH grapefruits, which was almost devoid of carotenoids and with far few chromoplasts per cell, the chromoplast crystalloid structures were never detected and it was also noticeable the lack of well-defined structures, with the only presence of low electron-dense lipid droplets and scarce achlorophyllous membranes (Fig. 6a, e, f).

Discussion

Over decades, growers and breeding programmes have used the genetic diversity among the *Citrus* genus to select new cultivars as well as spontaneous and induced mutations differing in either external or internal fruit quality, in which colouration is one of the most remarkable selection parameters (Kato 2012; Garcia-Lor et al. 2013; Rodrigo et al. 2013). As carotenoids are the main pigments in mature citrus fruits, a great diversity of carotenoid profiles has been created, which constitute an excellent experimental material to explore the biochemical and molecular bases governing citrus fruit colouration.

In all varieties selected for this study, carotenoid content was at least three times higher in the peel than in the pulp (Figs. 1, 4), in agreement with previous reports (Xu et al. 2006; Alquézar et al. 2008, 2013; Kato 2012). Moreover, the qualitative composition of carotenoids was also different between both tissues, reinforcing the notion of an independent regulation of carotenoid biogenesis (Alquézar et al. 2008; Matsumoto et al. 2009), which may also be related to the absence of communication or metabolite exchange between peel and pulp in citrus fruit (Tadeo et al. 2008). Therefore, this carotenoid tissue-specific diversity was of special relevance to investigate the changes

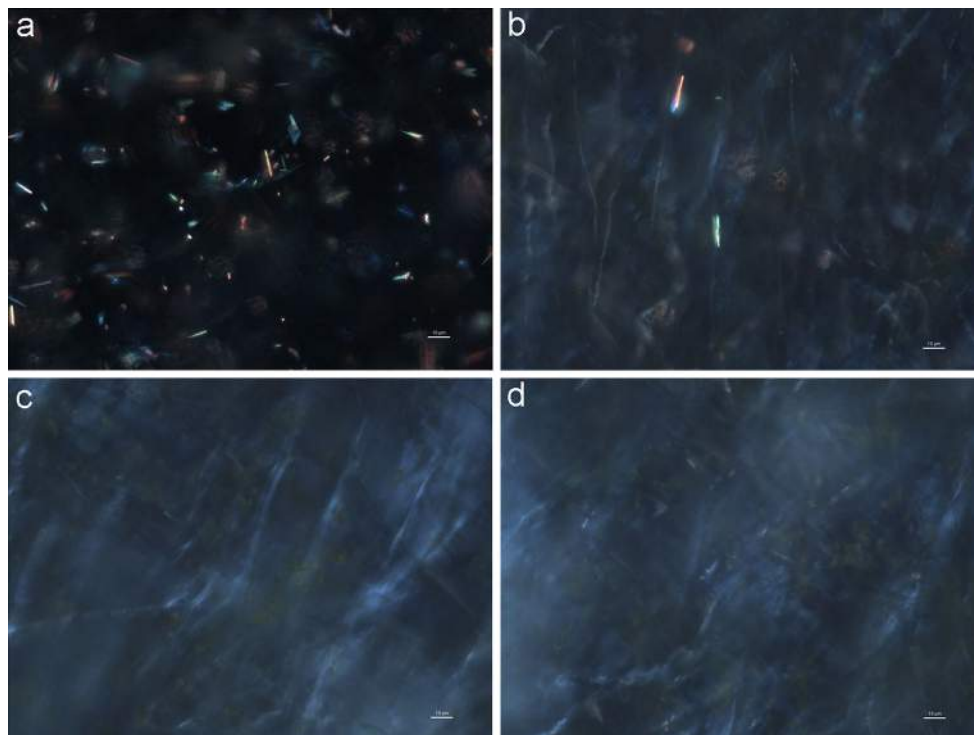


Fig. 7 Polarized light microscopy images of the pulp of the red SR grapefruit (**a**), the red C orange mutant (**b**), the yellow P orange mutant (**c**) and the ordinary N orange (**d**) from full-coloured fruits.

Note the presence of lycopene crystals only in pulp of red varieties, highly abundant in SR grapefruit (**a**) and more scarcely in C orange (**b**)

occurring at the plastidial level, which encompass the evolution in carotenoid composition during ripening.

In the peel of ordinary sweet orange, the content of characteristic chloroplastic carotenoids (lutein, α - and β -carotene, zeaxanthin and neoxanthin) is normally reduced during the colour change, while the synthesis of β , β -xanthophylls is amplified, accumulating mainly 9-(Z)-violaxanthin (Kato et al. 2004; Rodrigo et al. 2004, 2013; Kato 2012). Chloroplastic-type carotenoids were detected in the fruit peel at breaker but not at full colour stage (Fig. 1), and the main plastidial structures observed in the peel at this stage were stacked thylakoids arranged in typical chloroplastic grana with associated starch grains (Figs. 2a–f, 3a–d), resembling the plastid organization described in citrus leaves (Freeman et al. 1978). Only plastids in an advanced stage of transition from chloro- to chromoplasts showed disassembly of thylakoid membranes, starch grain disappearance and the gradual development of the different sink structures associated with the accumulation of newly synthesized carotenoids (Fig. 2a, f).

In agreement with previous reports (Rodrigo et al. 2003; Alquézar et al. 2008, 2013), differences in carotenoid composition were already observed among the five varieties in the fruit peel at the breaker stage (Fig. 1a, b); however, only minor variations in chloroplast ultrastructure were observed (Figs. 2a–f, 3a–d). Thus, it appears that chloroplasts from these citrus varieties have somehow the ability to sequester significant amounts of unusual carotenes without greatly affecting plastid structure. This fact could be directly related to the presence of dense thylakoidal membranes in these plastids, which have been demonstrated as an active site for carotenoids biosynthesis and storage in other fruit (Nogueira et al. 2013).

A feature observed in orange peel plastids at breaker stage was the presence of starch grains (Fig. 2a, b, e). It has been reported that P mutant has 3–4 times higher starch content compared to its parental line (Holland et al. 2005), and starch grains size and number notably increased in the yellow P mutant as fruit ripened, which was less noticeable in N and C plastids (Suppl. Fig. S1). This alteration in starch accumulation and sugar metabolism in P may be linked to changes to the subplastidial structures and carotenoid composition (Fig. 1c), as has been recently reported in transgenic tomato fruit where a higher content of upstream carotenes was also related to changes in sugars, amino acids and organic acids levels (Nogueira et al. 2013).

In the pulp tissue of all the varieties studied, the ultrastructural transition from breaker to full colour was less noticeable, since no chloroplasts were detected in this tissue and changes mainly consisted in the appearance of new carotenoid-sequestering structures (Cazzonelli and Pogson 2010; Li and Yuan 2013). Therefore, the absence of pre-

existing chloroplasts in citrus pulp cells suggests a direct differentiation from proplastids, similarly to what occurs in papaya (Schweiggert et al. 2011) and peach palm tropical fruit (Hempel et al. 2014). In contrast to the observations in peel tissue, important differences in plastid ultrastructure among varieties were already detectable in the pulp at breaker stage (Figs. 5a–f, 6a–d), coinciding with a contrasting carotenoid composition (Fig. 4a, b). The noticeable differences in plastid ultrastructure between peel and pulp tissues at the breaker stage could be related to the scarce presence of membranes in the plastids from the juice vesicle cells, and therefore, with an earlier need to develop new deposition sites to sustain carotenoid accumulation.

The presence of starch grains at the breaker stage was restricted to plastids from the orange mutants P and C (Fig. 5c–e), whereas they were neither observed in N orange nor in MSH and SR grapefruit pulps (Figs. 5a–b, 6a–d). Differences in the pulp chromoplasts at the breaker stage were related to the presence of achlorophyllous membranes in N orange and MSH grapefruit (Figs. 5a, b, 6b), since they were not detected in any of the other varieties, and they disappeared as the fruit ripened (Fig. 5g, h). The reason for this difference remains to be elucidated but could be somehow linked to the lack of starch grains observed in N and MSH plastids. Another distinctive feature of C orange and SR grapefruit pulp chromoplasts was the presence of needle-like crystals (Figs. 5f, l, 6c, d, g, h), coinciding with the presence of lycopene in this tissue and resembling those previously described in other lycopene-accumulating fruits (Simkin et al. 2007; Schweiggert et al. 2011; Jeffery et al. 2012; Nogueira et al. 2013) and in the pulp and peel of red grapefruit (Cao et al. 2012; Jeffery et al. 2012; Lado et al. 2015). By contrast, at breaker stage in MSH pulp, the low content of carotenoids was related to a lack of any defined sink structures (Fig. 6a, b).

The differences found in the pulp plastids at the breaker stage of the five varieties analysed were amplified in ripe fruit, coinciding with major differences in carotenoid composition (Fig. 4c, d). The pulp of full-coloured C and P fruit had at least 6 times more carotenoids than the parental N, with extremely high quantities of the linear phytoene in P and C pulp, a moderate content of phytofluene and ζ -carotene in P, and lycopene in C (Fig. 4c). The pulp of SR grapefruit accumulated 40 times more carotenoids than MSH, and the presence of phytoene and lycopene was pronounced (Fig. 4d). The composition of carotenoids in P pulp was associated with the presence of a novel plastid type containing electron-lucent, smooth and round vesicles (Fig. 5i). These kinds of round even vesicles were similar to that described in an *Arabidopsis* mutant with a disruption in the phytoene desaturase gene leading to the accumulation of high amounts of phytoene (Qin et al. 2007), and to those described in peach palm (*Bactris gasipaes*),

accumulating the rare γ -carotene (30 % of total carotenoids; Hempel et al. 2014). Therefore, this type of vesicle seems to be related to the massive accumulation of linear carotenoids in P juice vesicle cells. Interestingly, these even round vesicles abundant in P pulp were not observed in any of the peel chromoplasts of the three orange varieties studied; instead, huge uneven lipid droplets (LD) were detected in P peel chromoplasts (Fig. 2i, j), but the composition of these LD remains to be determined. These LD were organized inside the chromoplasts or even released from the plastids into the cytosol, probably when reaching certain size or abundance thresholds (Fig. 2i, Suppl. Fig. S3). It has been recently suggested that compartmentalization of carotenoids could be a cellular mechanism to remove excess carotenoids accumulated in membranes or inside plastids to alleviate potential cell damaging effects in carotenoid-rich tissues (Nogueira et al. 2013). The presence of similar lipid bodies in the cell cytoplasm has been previously described in chili pepper (Liu 2013) and in avocado fruit (Platt-Aloia and Thomson 1981), where they may coalesce and form larger lipid bodies in the cytoplasm similar to those observed in oranges. Lipid-droplet structures were observed in the sections prepared from the peel of the three oranges studied (Suppl. Figs. S4 and S5); however, they were more abundant in P mutant and hardly observed in N orange (Suppl. Fig. S3), coinciding with the differences in carotenoid content and composition observed between these varieties (Fig. 1c). Moreover, these LD were not found in MSH and SR grapefruit (Fig. 6), which showed a reduced total carotenoid content in this tissue compared to oranges.

Plastoglobuli were present in pulp chromoplasts of all varieties, with the exception of MSH grapefruit (Figs. 5, 6), and constitute a key site for carotenoid biosynthesis and deposition in plants, being a common structure present in coloured carotenoid-accumulating tissues (Ytterberg et al. 2006; Br  h  lin et al. 2008; Li and Yuan 2013). Plastoglobuli are linked to membranes, since they were proposed to be developed attached to thylakoids and other plastidial membranes and afterwards released into the stroma (Ytterberg et al. 2006; Br  h  lin et al. 2008). Plastoglobuli represent an excellent carotenoid-sequestering sink with at least two objectives: to alleviate potential damaging effects from carotenoid accumulation in membranes and to avoid further desaturation reactions as a way of regulating flux of the pathway by partitioning carotenoid precursors from their biosynthetic enzymes (Nogueira et al. 2013). Interestingly, typical plastoglobuli were not found in P mutant peel chromoplasts (Fig. 2i, j), but were otherwise abundant in C and N (Fig. 2g, k). It has been proposed that plastoglobuli constitute the main site for xanthophyll accumulation in oranges (Thomson 1966; Gross 1987; Zeng et al. 2011), and the presence of

plastoglobuli has also been reported in other citrus such as pummelo (Gross et al. 1983), kumquat (Huyskens et al. 1985), Valencia oranges (Thomson 1966), Satsuma mandarins (Shimokawa et al. 1978) and lemon (Ljubecic 1984). Our results are in agreement with these observations, since both N and C peel accumulated high content of xanthophylls (more than 70 % of total carotenoid content), while they only represented 10 % of total carotenoids in fully ripen P (Fig. 1c). Apart from xanthophylls, these plastoglobuli have recently been demonstrated to accumulate phytoene in tomato (Nogueira et al. 2013). In this sense, it is worth noting that in MSH grapefruit peel, with low carotenoid content but nearly 80 % being phytoene and xanthophylls, and in SR, rich in phytoene (30 %), the predominant sub-chromoplast structures were also plastoglobuli (Figs. 3e, f, 6g, h). Therefore, plastoglobuli could represent an important sink for phytoene and xanthophylls accumulation in citrus fruit. Moreover, it is well known that the main xanthophylls in ripening citrus fruits are esterified with fatty acids conferring a greater compound stability (Gross 1987). In mature fruit tissues of sweet oranges and grapefruits, the percentage of xanthophylls esterified is variable, but can reach up to 85–90 % of the total (Philip 1973a, b; Gross 1987; Giuffrida et al. 2010). Recently, it has been demonstrated that plastoglobuli are a main site for accumulation of esterified xanthophylls in chromoplast tissues (Ariizumi et al. 2014).

The presence of achlorophyllous membranes in the full-coloured flavedo chromoplasts of all varieties could be related to the need for an effective anchor site for protein complexes where carotenoid biosynthesis eventually takes place, as has been reported in tomato (Nogueira et al. 2013) and pepper (Deru  re et al. 1994). Similar membranes were described in the chromoplast from pummelo (Gross et al. 1983). Chemical content and composition of such membranes have been investigated, containing mainly lipids (mostly galactolipids), carotenoids (like phytoene or β -carotene) and certain proteins such as fibrillins or plastoglobulins, as well as key carotenoid biosynthetic enzymes (Gross 1987; Nogueira et al. 2013). Therefore, membranes could represent a relevant structure necessary for carotenoid biosynthesis in the chromoplasts of citrus fruit peel, since their presence was usually related to an increase in carotenoid content.

Accumulation of (*all-E*)-lycopene in the pulp of C orange and SR grapefruit was linked to the detection of needle-shaped crystalloid structures, surrounded by membrane remains due to fixation and sample preparation (Figs. 5f, l, 6c, g, h), that were confirmed by polarized light microscopy and varied in size from 2 to 14 μm (Fig. 7a, b). These crystals could facilitate lycopene accumulation and limit isomerization reactions, as has been proposed for (*all-E*)-isomers of β -carotene in carrot roots (Kim et al. 2010), mango

Table 1 Summary of the main plastid types and substructures identified in the peel and pulp cells of the different citrus varieties at breaker and full colour ripening stages

Specie	Variety	Peel		Pulp	
		Breaker	Full colour	Breaker	Full colour
Orange	Navel	Chl-Chm (Pg, s, t)	GM (Pg , s, mm,)	GM (Pg , mm)	GM (Pg)
	Pinalate	Chl-Chm (Pg, s, t)	GM (LD , s, mm,)	GM (Pg, s)	GM (Pg); V (v)
	Cara Cara	Chl-Chm (Pg, s, t)	GM (Pg , s, mm)	GM (Pg, s); CR (c)	GM (Pg, s); CR (Pg, c)
Grapefruit	MSH	Chl-Chm (Pg, s, t)	GM (Pg , mm)	GM (mm, Igd)	GM (Igd)
	SR	Chl-Chm (Pg, s, t)	GM (Pg , mm, Igd)	CR (Pg, c)	GM (Pg); CR (Pg, c)

Plastid type: *Chl-Chr* plastids in transition from chloro- to chromoplast, *GM* globular-membranous chromoplast, *CR* crystalloid chromoplast, *V* novel type of chromoplast full of even and round vesicles. The main substructures identified in the plastids are indicated in parenthesis, and when a structure is predominant, is indicated in bold. *Pg* plastoglobuli, *s* starch grains, *t* thylakoids, *c* lycopene crystals, *mm* achlorophyllous membranes, *LD* lipid droplets, *Igl* irregular low electron-dense droplets, *v* even and round vesicles

(Vasquez-Caicedo et al. 2006; Jeffery et al. 2012) and (*all-E*)-lycopene in tomato (Simkin et al. 2007; Nogueira et al. 2013). Carotenoid-containing crystals were firstly described to occur inside the lumina of some thylakoids, exhibiting an external membrane even in mature chromoplasts (Ljubesic et al. 1991). However, more recently, crystalloid structures have been described to involve invagination of the inner envelope of the plastid, indicating that the newly synthesized membranes are not dependent on pre-existing thylakoids (Simkin et al. 2007). Therefore, the presence of a previous thylakoidal membrane is not a prerequisite for crystal formation in citrus fruit, although the existence of surrounding membranes was always linked to lycopene crystals in SR and C pulp (Figs. 5l, 6g, h). It is worth noting that despite the similar lycopene concentration in the pulp of C mutant and SR grapefruit (Fig. 4c, d), TEM micrographs and visualization of juice vesicle membranes by polarized light microscopy revealed a higher crystal abundance in SR pulp (Fig. 7a, b). The reason for this remains to be elucidated but could be related to possible differences in the mechanisms that lead to lycopene accumulation in both varieties (Alquézar et al. 2008, 2013) and to the relative importance of lycopene in relation to other carotenoids, accounting for 48 % in the pulp of full-coloured Star Ruby and for 19 % in C orange mutant (Fig. 4c, d). Moreover, it has been recently demonstrated that in peach palm fruit, β -carotene, lycopene and γ -carotene occur predominantly in lipid-dissolved and not in crystalline form (Hempel et al. 2014). Therefore, it is tempting to speculate that in C pulp, lycopene may be partially deposited as lipid-dissolved structures influenced by the presence of other accompanying-carotenoids, mostly xanthophylls.

According to the carotenoid differences between the citrus varieties considered in this study, chromoplast ultrastructure in the flavedo and pulp tissues was strongly influenced by quantitative composition and the presence of individual carotenoids. A summary of the main plastid types

and substructures identified in the peel and pulp tissues of the different citrus varieties is presented in Table 1. At full colour stages, plastoglobuli were the commonest structure found in citrus chromoplasts, both in the peel and the pulp, with the exception of P and MSH. In this sense, plastoglobuli and achlorophyllous membranes were the main structures for carotenoid accumulation in mature citrus fruit, while (*all-E*)-lycopene was preferably stored inside crystalline structures in the red pulp of SR grapefruit and C sweet orange. The former allows the classification of citrus chromoplasts into a globular-membranous type, while the presence of certain carotenoids (such as lycopene) correlates with the development of crystalloid chromoplast. Similarly, alterations in carotenoid complement found in the citrus orange mutant P of pale-yellow colouration lead to the development of a novel vesicular-type chromoplasts with even and round subchromoplast membrane-bound vesicular structures most likely containing high amounts of linear carotenes. In this sense, it is worth to mention that to our knowledge the peel of P fruits contains the highest carotenoid concentration reported in a citrus fruit (nearly 400 $\mu\text{g g}^{-1}$ FW) and one of the top in phytoene among fruits (263 $\mu\text{g g}^{-1}$ FW) (Meléndez-Martínez et al. 2015). Therefore, we conclude that citrus plastids have an extraordinary plasticity to rearrange carotenoids inside different compartments to allow massive pigment accumulation, probably as a mechanism to avoid potential harmful effects. Future work should be focused on investigating the molecular and biochemical mechanisms governing these changes in the different citrus varieties.

Author contribution statement JL performed the experiments, carotenoids extraction and quantification as well as the preparation of samples and visualization at the microscope. JL was in charge of writing the manuscript. LZ and MJR designed and supervised the research and manuscript redaction. AG participated in carotenoids analyses. AP and

AS were involved in discussion, interpretation and supervision of microscopy work during JL stay at Biomedical Imaging Unit, Southampton University and Royal Holloway, University of London. All authors have read and approved the manuscript.

Acknowledgments This work was supported by research grants AGL2009-11558 and AGL2012-34573 (Ministerio de Economía y Competitividad, Spain). Financial support from PROMETEO 2014/0027 (Generalitat Valenciana) is also acknowledged. We acknowledge the use of the Spanish Citrus Germplasm Bank (IVIA, Moncada, Valencia, Spain) and Dr. Gema Ancillo for experimental material. The technical assistance of Amparo Beneyto is gratefully acknowledged. J. Lado was recipient of a JAE-Predoc (CSIC-European Social Fund) contract and a fellowship (JAE-CSIC) to conduct microscopy experiments at the Biomedical Imaging Unit, University of Southampton (UK). JL, MJR and LZ are members of the network IBERCAROT funded by CYTED (Ref. 112RT0445).

References

- Alquézar B, Rodrigo MJ, Zacarías L (2008) Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant ‘Cara Cara’. *Phytochemistry* 69:1997–2007
- Alquézar B, Rodrigo MJ, Lado J, Zacarías L (2013) A comparative physiological and transcriptional study of carotenoid biosynthesis in white and red grapefruit (*Citrus paradisi* Macf). *Tree Genet Genomes* 9:1257–1269
- Ariizumi T, Kishimoto S, Kakami R, Maoka T, Hirakawa H, Suzuki Y, Ozeki Y, Shirasawa K, Bernillon S, Okabe Y et al (2014) Identification of the carotenoid modifying gene *PALE YELLOW PETAL 1* as an essential factor in xanthophyll esterification and yellow flower pigmentation in tomato (*Solanum lycopersicum*). *Plant J* 79:453–465
- Berman J, Zorrilla-López U, Farré G, Zhu C, Sandmann G, Twyman RM, Capell T, Christou P (2014) Nutritionally important carotenoids as consumer products. *Phytochem Rev*. doi:10.1007/s11101-014-9373-1
- Bréhélin C, Kessler F, Van Wijk KJ (2008) Plastoglobules: versatile lipoprotein particles in plastids. *Trends Plant Sci* 12:260–266
- Britton G (1995) Carotenoids, vol. 1B: Spectroscopy. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids. Basel, Birkhäuser, pp 13–63
- Britton G (2008) Carotenoids, vol. 4: natural functions. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids. Basel, Birkhäuser, pp 189–212
- Burns JK, Achor DS, Echeverria E (1992) Ultrastructural studies on the ontogeny of grapefruit juice vesicles (*Citrus paradisi* Macf cv Star Ruby). *Int J Plant Sci* 153:14–25
- Cao H, Zhang J, Xu J, Ye J, Yun Z, Xu Q, Xu J, Deng X (2012) Comprehending crystalline β -carotene accumulation by comparing engineered cell models and the natural carotenoid-rich system of citrus. *J Exp Bot* 63:4403–4417
- Cazzonelli CI, Pogson BJ (2010) Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci* 15:266–274
- Deruère J, Römer S, D’Harlingue A, Backhaus RA, Kuntz M, Camara B (1994) Fibril assembly and carotenoid overaccumulation in chromoplast: a model for supramolecular lipoprotein structures. *Plant Cell* 6:119–133
- Egea I, Barsan C, Bian W, Purgatto E, Latché A, Chervin C, Bouzayen M, Pech JC (2010) Chromoplast differentiation: current status and perspectives. *Plant Cell Physiol* 51:1601–1611
- Fanciullino AL, Dhuique-Mayer C, Luro F, Casanova J, Morillon R, Ollitrault P (2006) Carotenoid diversity in cultivated citrus is highly influenced by genetic factors. *J Agric Food Chem* 54:4397–4406
- Freeman BA, Platt-Aloia K, Mudd JB, Thomson WW (1978) Ultrastructural and lipid changes associated with the aging of citrus leaves. *Protoplasma* 94:221–233
- García-Lor A, Curk F, Snoussi-Trifa H, Morillon R, Ancillo G, Luro F, Navarro L, Ollitrault P (2013) A nuclear phylogenetic analysis: SNPs, indels and SSRs deliver new insights into the relationships in the ‘true citrus fruit trees’ group (Citrinae, Rutaceae) and the origin of cultivated species. *Ann Bot* 111:1–19
- Giuffrida D, Dugo P, Salvo A, Saitta M, Dugo G (2010) Free carotenoid and carotenoid ester composition in native orange juices of different varieties. *Fruits* 65:277–284
- Gmitter FJ (1995) Origin, evolution and breeding of the grapefruit. In: Janick J (ed) Plant breeding reviews. Wiley, Florida, pp 345–363
- Gross J (1987) Pigments in fruits. In: Schweigert BS (ed) Food science and technology: a series of monographs. Academic Press, London
- Gross J, Timberg R, Graef M (1983) Pigment and ultrastructural changes in the developing pummel *Citrus grandis* ‘Goliath’. *Bot Gaz* 144:401–406
- Hempel J, Amrehn E, Quesada S, Esquivel P, Jiménez VM, Heller A, Carle R, Schweiggert RM (2014) Lipid-dissolved γ -carotene, β -carotene, and lycopene in globular chromoplasts of peach palm (*Bactris gasipaes* Kunth) fruits. *Planta* 240:1037–1050
- Holland N, Menezes HC, Lafuente MT (2005) Carbohydrate metabolism as related to high-temperature conditioning and peel disorders occurring during storage of citrus fruit. *J Agric Food Chem* 53:8790–8796
- Huyskens S, Timberg R, Gross J (1985) Pigment and plastid ultrastructural changes in kumquat (*Fortunella margarita*) Nagami during ripening. *J Plant Physiol* 118:61–72
- Jeffery J, Holzenburg A, King S (2012) Physical barriers to carotenoid bioaccessibility ultrastructure survey of chromoplast and cell wall morphology in nine carotenoid-containing fruits and vegetables. *J Sci Food Agric* 92:2594–2602
- Kato M (2012) Mechanism of carotenoid accumulation in citrus fruit. *J Jpn Soc Hortic Sci* 81:219–233
- Kato M, Ikoma Y, Matsumoto H, Sugiura M, Hyodo H, Yano M (2004) Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in *Citrus* fruit. *Plant Physiol* 134:824–837
- Kim JE, Rensing KH, Douglas CJ, Cheng KM (2010) Chromoplasts ultrastructure and estimated carotene content in root secondary phloem of different carrot varieties. *Planta* 23:549–558
- Lado J, Cronje P, Alquézar B, Page A, Manzi M, Gomez-Cadenas A, Stead AD, Zacarías L, Rodrigo MJ (2015) Fruit shading enhances peel colour, carotenes accumulation and chromoplast differentiation in red grapefruit. *Physiol Plant*. doi:10.1111/ppl.12332
- Lee HS (2001) Characterization of carotenoids in juice of red navel orange (Cara Cara). *J Agric Food Chem* 49:2563–2568
- Li L, Yuan H (2013) Chromoplast biogenesis and carotenoid accumulation. *Arch Biochem Biophys* 539:102–109
- Liu L (2013) Ultrastructural study on dynamics of lipid bodies and plastids during ripening of chili pepper fruits. *Micron* 46:43–50
- Ljubecic N (1984) Structural and functional changes of plastids during yellowing and regreening of lemon fruits. *Acta Bot Croat* 43:25–30
- Ljubecic N, Wrisher M, Devise Z (1991) Chromoplasts—the last stages in plastid development. *Int J Dev Biol* 35:251–258
- Matsumoto H, Ikoma Y, Kato M, Nakajima N, Hasegawa Y (2009) Effect of postharvest temperature and ethylene on carotenoid

- accumulation in the flavedo and juice sacs of Satsuma mandarin (*Citrus unshiu* Marc). *J Agric Food Chem* 57:4724–4732
- Meléndez-Martínez AJ, Mapelli-Brahm P, Benítez-González A, Stinco CM (2015) A comprehensive review on the colorless carotenoids phytoene and phytofluene. *Arch Biochem Biophys* 572:188–200
- Nogueira M, Mora L, Enfissi EM, Bramley PM, Fraser PD (2013) Subchromoplast sequestration of carotenoids affects regulatory mechanisms in tomato lines expressing different carotenoid gene combinations. *Plant Cell* 25:4560–4579
- Oberholster R, Cowan AK, Molnár P, Tóth G (2001) Biochemical basis of color as an aesthetic quality in *Citrus sinensis*. *J Agric Food Chem* 49:303–307
- Philip T (1973a) Nature of xanthophyll esterification in grapefruits. *J Agric Food Chem* 21:963–964
- Philip T (1973b) The nature of carotenoid esterification in citrus fruits. *J Agric Food Chem* 21:964–966
- Platt-Aloia KA, Thomson WW (1981) Ultrastructure of the mesocarp of mature avocado fruit and changes associated with ripening. *Ann Bot* 48:451–465
- Qin G, Gu H, Ma L, Peng Y, Deng XW, Chen Z, Qu LJ (2007) Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in *Arabidopsis* by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. *Cell Res* 17:471–482
- Rodrigo MJ, Marcos JF, Alférez F, Mallent MD, Zacarías L (2003) Characterization of Pinalate, a novel *Citrus sinensis* mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. *J Exp Bot* 54:727–738
- Rodrigo MJ, Marcos JF, Zacarías L (2004) Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L) during fruit development and maturation. *J Agric Food Chem* 52:6724–6731
- Rodrigo MJ, Alquézar B, Alós E, Lado J, Zacarías L (2013) Biochemical bases and molecular regulation of pigmentation in the peel of *Citrus* fruit. *Sci Hort* 163:42–62
- Saunt J (2000) *Citrus varieties of the world*. Sinclair International Limited, Norwich
- Schweiggert RM, Steingass CB, Heller A, Esquivel P, Carle R (2011) Characterization of chromoplasts and carotenoids of red- and yellow-fleshed papaya (*Carica papaya* L). *Planta* 234:1031–1044
- Shimokawa K, Sakanoshita A, Horiba K (1978) Ethylene-induced changes of chloroplast structure in Satsuma mandarin (*Citrus unshiu* Marc). *Plant Cell Physiol* 19:220–236
- Simkin AJ, Gaffé J, Alcaraz JP, Carde JP, Bramley PM, Fraser PD, Kuntz M (2007) Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. *Phytochemistry* 68:1545–1556
- Sitte P, Falk H, Liedvogel B (1980) Chromoplasts. In: Czygan FGC (ed) *Pigments in plants*. Fischer Verlag, Stuttgart, pp 117–148
- Smith JHC, Benítez A (1955) Chlorophylls: analysis in plant materials, vol. 4. In: Paech K, Tracey MV (eds) *Moderne Methoden der Pflanzenanalyse*. Springer, Berlin, pp 142–196
- Tadeo FR, Cercos M, Colmenero-Flores JM, Iglesias DJ, Naranjo MA, Rios G, Carrera E, Ruiz-Rivero O, Lliso I, Morillon R, Ollitrault P, Talon M (2008) Molecular physiology of development and quality of citrus. *Adv Bot Res* 47:147–223
- Thomson WW (1966) Ultrastructural development of chromoplasts in Valencia oranges. *Bot Gaz* 127:133–139
- Vasquez-Caicedo AL, Heller A, Neidhart S, Carle R (2006) Chromoplast morphology and β -carotene accumulation during postharvest ripening of mango cv ‘Tommy Atkins’. *J Agric Food Chem* 54:5769–5776
- Vishnevetsky M, Ovadis M, Vainstein A (1999) Carotenoid sequestration in plants: the role of carotenoid-associated proteins. *Trends Plant Sci* 4:232–235
- Xu CJ, Fraser PD, Wang WJ, Bramley PM (2006) Differences in the carotenoid content of ordinary citrus and lycopene-accumulating mutants. *J Agric Food Chem* 54:5474–5481
- Ytterberg AJ, Peltier JB, van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. *Plant Physiol* 140:984–997
- Zeng Y, Pan Z, Ding Y, Zhu A, Cao H, Xu Q, Deng X (2011) A proteomic analysis of the chromoplasts isolated from sweet orange fruits (*Citrus sinensis* (L) Osbeck). *J Exp Bot* 62:5297–5309