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Carotenoid Biosynthesis and their Regulation in Citrus Fruits

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ABSTRACT

Carotenoids are the main pigments responsible for the attractive color of the peel and pulp of citrus fruits and greatly contribute to their nutritional and antioxidant value. Fruits of different *Citrus* species display a broad array of color singularities and in many cultivars the peel and the pulp also exhibit different color, envisaging specie- and tissue- specific regulation of the carotenoid content and composition. In addition, citrus mutants affected in fruit color are a useful experimental system to identify molecular mechanisms regulating carotenoid biosynthesis. *Citrus* is, therefore, an excellent model to study fruit carotenoid accumulation and their regulation. In this review, we summarize and update information on carotenoid content and composition in fruits of agronomically important *Citrus* species. Current understanding of carotenoids biosynthesis in citrus, highlighting the main regulatory steps of the pathway and how may be related to carotenoid content and complement in peel and pulp of *Citrus* fruits are discussed. Finally, the effect of environmental and endogenous factors on citrus fruits carotenoids is also evaluated.

Keywords: color, carotenogenesis, gene expression, maturation

Abbreviations: ABA, abscisic acid; **β-CHX**, β-carotene hydroxylase; **ε-CHX**, ε-carotene hydroxylase; **CRTISO**, carotene isomerase; **GA**, gibberellic acid; **GGPP**, geranylgeranyl diphosphate; **β-LCY**, lycopene β-cyclase; **ε-LCY**, lycopene ε-cyclase; **MEP**, 2-C-methyl-D-erythritol 4-phosphate; **NCED**, nine-*cis*-epoxycarotenoid dioxygenase; **NSY**, neoxanthin synthase; **PDS**, phytoene desaturase; **PSY**, phytoene synthase; **PTOX**, plastid terminal oxidase; **VDE**, violaxanthin de-epoxidase; **ZDS**, ζ-carotene desaturase; **ZEP**, zeaxanthin epoxidase

CONTENTS

INTRODUCTION

Carotenoids were first described as isoprenoid compounds at the beginning of the 20th century (Gross 1987). Their basic structure is a C_{40} backbone skeleton, formed by tail to tail linkage of two geranylgeranyl diphosphate molecules. This skeleton can be modified by hydrogenation, dehydrogenation, cyclization, shortening or extension of the chain, isomerization, or by the addition of oxygen or other functional groups. Hydrocarbonated carotenoids are known as carotenes, while carotenoids that contain one or more oxygen atoms are referred to as xanthophylls. The most common oxygenated groups are hydroxy- and epoxy-, but aldehyde, ceto, carboxy, carbomethoxy and metoxy groups have been also described in carotenoids (Gross 1987). The structure of a linear, a bicyclic and an oxygenated carotenoid, corresponding to lycopene, β-carotene and violaxantin, respectively, are shown in **Fig. 1**.

Carotenoids are the pigments responsible of the red, yellow and orange colors of a wide number of flowers and fruits of many different plant species (Hirschberg 2001; DellaPenna and Pogson 2006). They also provide the characteristic coloration of many birds, fishes and crustaceous (pelicans, trout, salmon, lobsters, shrimp, etc.). However,

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Invited Review

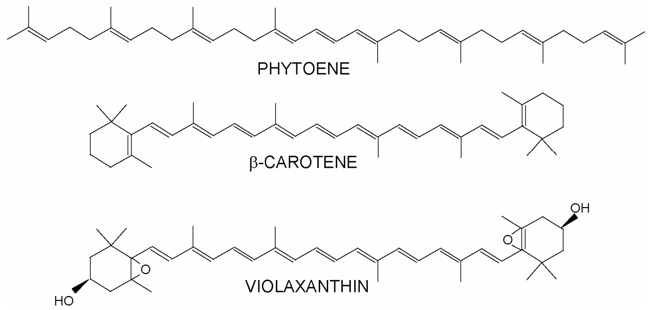

Fig. 1 Chemical structure of lineal (phytoene), bicyclic (β-carotene) and epoxy carotenoids (violaxanthin).

animals do not synthesize carotenoids and their color and carotenoid content depend on their dietary uptake (Fraser and Bramley 2004). In addition to these coloration-related properties, carotenoids have other major biological functions. Carotenoids act as modulators of the physical properties of lipid membranes (Gruszecki and Strzalka 2005), participate in photoprotection and in the light-harvesting complex of the photosynthetic apparatus (Demmig Adams *et al.* 1996; Merchant and Sawaya 2005) and play important roles as photoprotectives and scavengers of free radicals (Fraser and Bramley 2004). Moreover, carotenoids are precursor of apocarotenoids, some of them are important volatile aroma-related compounds (Bouvier *et al.* 2003; Simkin *et al.* 2004; Lewinsohn *et al.* 2005). The plant hormone abscisic acid (ABA) is also an apocarotenoid and, recently, new functions as signalling molecules have been described for some apocarotenoids (Schwartz *et al.* 2004). Their importance as nutritional components are also well recognized, since specific carotenoids are precursors of vitamin A, have significant antioxidant activity and protective effects against age-related ocular diseases, carcinogenesis, cardiovascular diseases and other degenerative processes (Fraser and Bramley 2004; Krinsky and Johnson 2005; Rao and Rao 2007; Renzi and Johnson 2007).

Carotenoids are widely distributed in the plant kingdom and their content and relative proportion varies greatly among the different species, tissue, developmental processes, cultural conditions, etc. In green vegetables and green tissues, the most abundant carotenoids are those characteristic of chloroplastic tissues, as lutein, zeaxanthin and β-carotene (Fraser and Bramley 2004; Niizu and Rodríguez Amaya 2005; Kopsell and Kopsell 2006). In fruits, carotenoid composition is much more complex and heterogeneous (Gross 1987). Due to the health promoting benefits of carotenoids, their use as foodstuff or as nutraceuticals has considerably increased last years. Carotenoid biosynthetic pathway has been studied in many plant species, most genes of the pathway have been identified and biotechnological manipulation of their content and composition has been performed in selected plant species (for review see Fraser and Bramley 2004; Bouvier *et al.* 2005; Botella-Pavía and Rodríguez-Concepción 2006; Howitt and Pogson 2006; Sandmann *et al.* 2006). Citrus fruits are one of the most important sources of carotenoids, because of the massive consumption worldwide as both fresh fruits and fruit juice. Carotenoid content and composition in fruits of different species and cultivars have been studied over the years, and more recently molecular analysis of the pathway has been also investigated. The objective of this review is to summarize current understanding of carotenoids biosynthesis in citrus, highlighting the main regulatory steps of the pathway and how may be related to the carotenoid content and complement in fruits of different *Citrus* species. The effect of environmental and endogenous factors on citrus fruits carotenoids is also evaluated.

CAROTENOIDS IN CITRUS

Citrus fruits are, in general, a complex source of carotenoids in which up to 110 different carotenes and xanthophylls have been reported, although many of them may be isomers (Stewart and Wheaton 1973a). These pigments are responsible for the external and internal coloration of fruit of most citrus species. In the commercialization of citrus fruits for fresh consumption, the external color is probably the first perception of the consumer and a critical factor for their acceptance. In the juice, the color is also and important attribute of quality. An interesting genetic feature of mature citrus fruit is the diversity of peel color which varies from the typical bright yellow of lemon, grapefruit or pummelo, to the characteristic orange of mandarins and oranges, or the red color of some grapefruit or pummelos mutants. This wide range of coloration is due to qualitatively and quantitatively changes not only in carotenoid content but also in their distribution. In the decade of 1960s, carotenoid content and composition were extensively studied by traditional chromatographic methods, as column chromatography and/ or thin layer chromatography (Curl and Bailey 1957a; Curl and Bayley 1961; Yokoyama and White 1966; Curl 1967; Yokoyama and White 1967; Yokoyama and Vandercook 1967) that provided valuable and fundamental information on chemistry of carotenoid. In 1971 Stewart and Wheaton demonstrated the potential of high performance liquid chromatography (HPLC) for the separation of saponified citrus carotenoids. HPLC coupled to photodiode array detector (PDA) offers a number of advantages, as short time of analysis, reproducibility, accuracy, low amount of samples, etc. and is currently the most widely used technique for carotenoid analysis, not only in citrus fruits but also in many other plant tissues and species.

Results collected from the decades of 1970-80 mainly provided correlations between peel or pulp color and carotenoid content and composition (Gross *et al.* 1972; Stewart 1977a, 1977b, 1980). From theses results it become evident that color in citrus fruits is highly related to total content of carotenoids but more importantly to specific carotenoids, that although may be at low concentrations have an important contribution in peel or juice color. More recently, nutritional values of citrus fruit carotenoids have been also evaluated (Lin and Chen 1994; Pupin *et al.* 1999; Lee 2001; Dhuique Mayer *et al.* 2005; Meléndez Martínez *et al.* 2005).

The diversity of citrus fruits carotenoids and the uncertain genetic origin of main cultivated species and varieties, made unattainable the understanding of the phylogenetic background regulating carotenoid content and composition. Exhaustive comparative analysis of carotenoid accumulation in fruit of different species and mutants, and their potential association with changes in gene expression are now generating new insights into this important metabolic pathway in citrus. Based on the carotenoid composition in the peel and juice sacs of 39 citrus cultivars during fruit ripening, Matsumoto *et al.* (2007) have established five clusters; carotenoid poor, phytoene-abundant, violaxanthin-abundant, violaxanthin- and β-cryptoxanthin-abundant, and phytoene-, violaxanthin-, and β-cryptoxanthin-abundant, respectively. This classification was identical for juice sacs with the exception of phytoene-abundant cluster, which was not detected in that tissue. It is interesting the fact that for most varieties, the carotenoid profile was qualitatively similar in peel and pulp (Matsumoto *et al.* 2007). Other authors suggest that *cis*-violaxanthin and β-cryptoxanthin content were strong determinants for the classification of 25 *Citrus* genotypes (Fanciullino *et al.* 2006). Different C_{30} citrus-specific carotenoids, referred to as apocarotenoids (**Fig. 2**), were also present in the peel and pulp of the fruit. One of the most abundant apocarotenoids β-citraurin, which is only present in the fruit peel and provides the intense orange-reddish coloration to some oranges, mandarins or hybrids (Stewart and Wheaton 1972; Stewart and Wheaton 1973b). In addition, other apocarotenoids, with longer (β-apo-2′-carotenal) or shorter (β-apo-14′-carotenal) chain have been

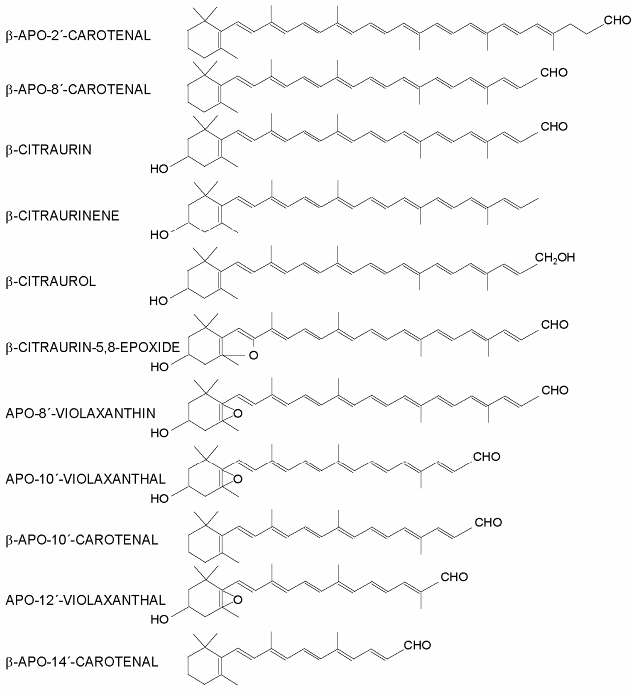

Fig. 2 Chemical structure of specific *Citrus* apocarotenoids and apocarotenals.

described. The biosynthetic origin of these aporacotenoids is still uncertain (Oberholster *et al.* 2001).

Oranges

The color of ripe orange fruits varies from the pale orange of Valencia orange to the intense orange of Navelina cultivar. Orange mutants of yellow (Rodrigo *et al.* 2003) or red color have been reported (Monselise and Halevy 1961; Lee 2001; Liu *et al.* 2007; Alquézar *et al.* 2008). Except for blood-oranges, in which the red color is due to the presence of anthocyanins (Lee 2002), the color of orange fruits is mainly due to carotenoids accumulation. Carotenoid content differs among cultivars, ranging from 4 to 38 µg/ g FW in the pulp and from 40 to 120 µg/ g FW in the peel. Similarly to most carotenogenic fruits, carotenoid content in orange fruit use to be higher in the peel than in the pulp (Gross 1987). Carotenoid content and composition in peel and pulp of important cultivated oranges in the world are summarized in **Table 1**. In ripe orange fruits, β,β-xanthophylls are the most abundant carotenoids that may represent up to 98% of total carotenoids in the flavedo of some cultivars (Rodrigo *et al.* 2004). Within these compounds, violaxanthin is the predominant and can reach up to 80% of total peel carotenoids. Violaxanthin 9-*cis*- isomer predominates in mature fruits, although the *trans*- and other *cis*- isomers may be also detected (Kato *et al.* 2004; Rodrigo *et al.* 2004; Meléndez Martínez *et al.* 2007). Violaxanthin renders a yellowish coloration and therefore is unlikely to be the only or major carotenoid responsible for the intense orange coloration of some varieties. Evidences indicate that the ratio between the content of yellow/orange carotenoids (as β-cryptoxanthin and β-citraurin) may be a better indicator of the typical coloration of orange and mandarins. For example, the ratio of violaxanthin/β-citraurin is lower in Navel oranges than in the pale Valencia (Oberholster *et al.* 2001) and the juice of Rhode Red had a lower violaxanthin/β-cryptoxanthin ratio than that of Valencia (Goodner *et al.* 2001).

Table 1 Carotenoid content and composition in the peel and pulp of mature fruits of selected orange (*Citrus sinensis* L. Osbeck) cultivars.

Cultivar	µg/g FW	Carotenoids	Reference
Bonanza			
Peel	43.3	31% auroxanthin 23% β-citraurin	Xu *et al.* 2006a
Pulp	6.6	33% violaxanthin 12% β-cryptoxanthin	Xu *et al.* 2006a
Cara Cara			
Peel	62.3	36% phytoene 21% auroxanthin	Xu *et al.* 2006a; Alquézar *et al.* 2008
Pulp	24.0-38.0	34-40% phytoene 8-14% violaxanthin 5-16% lycopene	Fanciullino *et al.* 2006; Xu *et al.* 2006a; Alquézar *et al.* 2008
Hamlin			
Pulp	4.0-10.0	36-57% violaxanthin 8-13% β-cryptoxanthin	Stewart 1977b; Dhuique Mayer *et al.* 2005
Navel			
Peel	60.0-75.0	26-52% violaxanthin 12% phytoene	Curl and Bayley 1961; Malachi *et al.* 1974; Alquézar *et al.* 2008
Pulp	4.0-23.0	48% violaxanthin 11% anteraxanthin	Curl and Bayley 1961; Gross *et al.* 1972; Alquézar *et al.* 2008
Navelate			
Peel	59.0	80% violaxanthin	Rodrigo *et al.* 2004
Navelina			
Peel	94.2	31% violaxanthin 18% phytoene	Pascual *et al.* 1993
Pulp	16.9		Pascual *et al.* 1993
Salustiana			
Pulp	8.6	55% violaxanthin 17% β-cryptoxanthin	Dhuique Mayer *et al.* 2005
Shamouti			
Pulp	5.9-27.7	38-47% violaxanthin 10-22% β-cryptoxanthin	Gross *et al.* 1972; Dhuique Mayer *et al.* 2005
Valencia			
Peel	90.0-120.0	44-55% violaxanthin	Gross 1987; Kato *et al.* 2004
Pulp	6.0-19.0	2-65% violaxanthin 4-15% β-cryptoxanthin 6-40% anteraxanthin	Stewart 1977b; Gross 1987; Kato *et al.* 2004; Dhuique Mayer *et al.* 2005

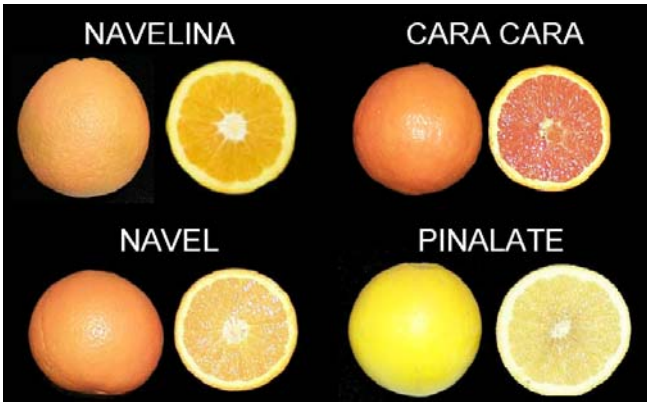

Fig. 3 External and internal appearance of mature orange (*Citrus sinensis* L. Osbeck) fruit with marked differences in peel and pulp color.

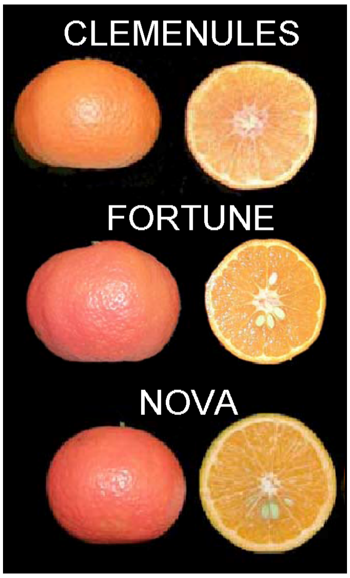

Fig. 4 External and internal appearance of mature fruits of Clemenules mandarin (*Citrus clementina*) and the hybrids Fortune (*Citrus clementina* x *Citrus reticulata*) and Nova (*Citrus clementina* x (*Citrus reticulata* x *Citrus paradisi*)).

Lycopene accumulation is an unusual feature in orange fruits, since has only been reported in three mutants. The orange Shara, a sport of Shamouti orange, develops a pink color at the inner mesocarp, carpel walls and vesicle starks, by accumulation of lycopene (Monselise and Halevy 1961). The orange Cara Cara was detected in Venezuela, as a bud mutation from Washington Navel, by its red-bright pulp that resembles that of pink and red grapefruits, while the peel remains orange (**Fig. 3**) (Saunt 2000). The red pulp of Cara Cara accumulates lycopene but also higher amounts of phytoene, phytofluene, and β-carotene than the parental fruit. In the peel of Cara Cara trace amounts of lycopene have been also detected (Lee 2001; Xu *et al.* 2006a; Alquézar *et al.* 2008). Cara Cara is the first lycopene-accumulating orange available after commercial propagation in California in 1991. Recently, a new red orange mutant, named Hong Anliu, accumulating lycopene in the albedo, membranes segment, and juice sacs, has been reported (Liu *et al.* 2007). This mutant, in contrast to Cara Cara, does not accumulate early carotenes, suggesting a different metabolic perturbation in the carotenoid biosynthetic pathway.

Another interesting mutant is Pinalate, obtained from Navelate orange in Spain, that is characterized by a bright yellow coloration in both peel and pulp, very similar to that of lemons and white grapefruits (**Fig. 3**). Carotenoid complement is however completely different in Pinalate fruits, since accumulated high levels of linear colorless carotenes (phytoene, phytofluene and ζ-carotene) and the concentration of xanthophylls is greatly reduced, generating thus a deficiency in ABA content in the peel. These data suggest that Pinalate may have a potential blockage at the level of ζ-carotene desaturation, either in ZDS or in ZDS-associated activities (Rodrigo *et al.* 2003).

Changes in carotenoid content and composition during fruit development and maturation have been studied in some orange varieties (Eilati *et al.* 1975; Kato *et al.* 2004; Rodrigo *et al.* 2004; Xu *et al.* 2006a; Matsumoto *et al.* 2007; Fanciullino *et al.* 2008). During the fruit green-stages, orange peel displayed a carotenoid profile characteristic of chloroplast-containing tissue, being lutein (ε,β-xanthophyll), *trans*-violaxanthin, β-carotene and neoxanthin the main carotenoids. During the transition form chloroplast to chromoplast, chlorophylls are progressively degraded and carotenoids decreased, to reach a minimum at which the fruit develops a yellow coloration just before the massive build up of carotenoids takes place. Fruit of most orange varieties accumulates β,β-xanthophylls, being 9-*cis*-violaxanthin the main carotenoid in the peel of mature fruit (**Table 1**). Carotenoid content in the pulp of immature orange fruit is very low and accumulated almost exclusively lutein and *trans*-violaxanthin. During fruit maturation, carotenoid content increased continuously but throughout the whole period was lower than in the peel. The major xanthophyll is also 9-*cis*-violaxanthin, which reached its maximum at the full-colored stage (Kato *et al.* 2004; Rodrigo *et al.* 2004; Alquézar *et al.* 2008).

Mandarins and its hybrids

Mandarins, also named as tangerines, are the most highly colored Citrus fruits, characterized by a bright orange-reddish color (**Fig. 4**). Mandarin fruits are very heterogeneous in terms of degreening rate and full coloration at maturity, since they comprise a large group of genetically different species and mutations. **Fig. 4** shows external and internal appearance of mature fruits of three typical mandarin varieties from the Clementine group: Clemenules, Fortune and Nova, where differences in fruit color are clearly noticeable.

The pattern of carotenoid accumulation in mandarin fruit is similar but more complex than that of orange fruit. At maturity more that twenty different carotenoids and xanthophylls can be detected in mandarin peel and pulp, and their content are also higher than in the corresponding tissue of oranges. **Table 2** summarizes carotenoid content and composition in peel and pulp of fruits from selected mandarin varieties. Total carotenoid content varies widely between cultivars, e.g. peel of Clementine mandarin contains four-times less total carotenoids than that of Satsuma. As in orange fruits, mandarins accumulated carotenoids mainly in the peel (up to 94% of total in Dancy mandarin) being β,β-xanthophylls the major constituents (68-90% of total carotenoids). The relative concentration of violaxanthin varied among cultivars and tissues, and the *cis*-isomer is by far more abundant than the *trans*-isomer.

Evolution of carotenoid content and composition during maturation of mandarin fruit is similar to that of orange fruit. In the peel of green fruit, chloroplastic carotenoids were the most abundant and after the color break increased the concentration of β,β-xanthophylls in both peel and pulp. A difference between mandarins and oranges is the higher accumulation of the xanthophyll β-cryptoxanthin and apocarotenoids in fruits of mandarins. β-Cryptoxanthin accumulates in both peel and pulp of mandarin fruit and contributes to the intense coloration of these tissues. The C_{30}-apocarotenoids β-citraurin and β-citraurinene are especially abundant in the peel where provide the distinctive orange-reddish pigmentation of mandarin fruits. The relative proportion of the red β-citraurin and the orange β-cryptoxanthin are thought to be the main contributors to the color in Dancy mandarin, even tough violaxanthin comprised more than half of total carotenoids (Gross 1987). Other example of deep coloration is the juice of Michal mandarin which appears to be related to high percentages of β-cryptoxanthin and zeaxanthin in pulp (Farin *et al.* 1983). It is interesting to mention that the differences in carotenoid composition between flavedo and pulp are more remarkable in mandarin than in oranges fruits (**Table 2**).

Table 2 Carotenoid content and composition in the peel and pulp of mature fruits of selected mandarin and mandarin hybrids cultivars.

Cultivar		µg/g FW	Carotenoids	Reference
Mandarin				
Clementine	Peel	74.6	32% β-citraurin 16% β-citraurinene	Farin *et al.* 1983
	Pulp	24.9	36% β-cryptoxanthin 35% violaxanthin	Fanciullino *et al.* 2006
Dancy	Peel	295.0	51% violaxanthin 8% β-citraurin	Farin *et al.* 1983
	Pulp	18.5	32% anteraxanthin 23% β-cryptoxanthin	Stewart 1977b
Satsuma	Peel	250.0-300.0	44% phytoene 19% β-cryptoxanthin 19% violaxanthin	Ikoma *et al.* 2001; Kato *et al.* 2004
	Pulp	21.0-34.0	55% β-cryptoxanthin 13% zeaxanthin	Ikoma *et al.* 2001; Kato *et al.* 2004; Fanciullino *et al.* 2006
Mandarin hybrids				
Hansen	Pulp	39.4	29% β-cryptoxanthin 19% zeaxanthin	Fanciullino *et al.* 2006
Michal	Peel	174.1	30% violaxanthin 26% β-citraurin	Farin *et al.* 1983
	Pulp	13.7	41% β-cryptoxanthin 9% zeaxanthin	Farin *et al.* 1983
Murcott	Pulp	44.9	33% β-cryptoxanthin 24% anteraxanthin 22% violaxanthin	Stewart 1977b
Orlando	Pulp	12.1	47% anteraxanthin 20% β-cryptoxanthin	Stewart 1977b
Robinson	Pulp	20.3	35% violaxanthin 28% β-cryptoxanthin	Stewart 1977b

Lemons

Lemon fruits are characterized by a typical yellow color, which may vary from a green-yellowish color in the pulp of Eureka to the intense yellow of Verna peel. The phylogenetic origin of lemons is unknown, but analysis using molecular markers suggests that may be an hybrid between citron and sour orange (Nicolosi *et al.* 2000).

During the transition from the green to the yellow stage, total carotenoid content decreased in the flavedo and pulp of lemon fruits, and at maturity the carotenoid compliment is less complex that in other Citrus species (Yokoyama and Vandercook 1967; Kato *et al.* 2004; Matsumoto *et al.* 2007). Linear colourless carotenes were the main pigments in the peel of lemon fruits; for example phytofluene accounted around 18 and 22% of total carotenoids in flavedo and pulp of Eureka lemons, respectively, and phytoene reached up to 86% of total carotenoids in Lisbon. Chloroplastic carotenoids were also presented at minor proportions, as lutein (around 15 µg/g FW), β-carotene (around 8 µg/g FW) and α-carotene (around 5 µg/g FW). In the pulp, the concentration of carotenoids is almost negligible (**Table 3**).

In fruits of other lemon varieties, as Meyer, Eureka Fr. and Volkamer, the most abundant carotenoid in the peel or pulp was β-cryptoxanthin (**Table 3**). Although the cause of this circumstance is not fully understood, it may be explained considering that these varieties are not true lemons, and may be originated from crosses with mandarins or oranges as parental, harbouring β-cryptoxanthin accumulation (Morton 1987; Nicolosi *et al.* 2000).

Pummelos

Pummelo or shaddock (*Citrus grandis* Osbeck) is considered one of the three true species of the genus Citrus. The fruit surpasses all other citrus fruit in size and, depending on the varieties, shows distinct morphological characteristics: as seedy or seedless, yellow, red and colorless (white). Carotenoid content and composition in these fruits had received little attention. **Table 4** summarizes results from studies conducted in ripe pummelo fruit of white (Goliath and Yuhuan)

Table 3 Carotenoid content and composition in the peel and pulp of mature fruits of selected lemon (*Citrus limon* Burm F.) cultivars.

Cultivar	µg/g FW	Carotenoids	Reference
Eureka			
Peel	1.4	18% phytofluene 17% ζ-carotene	Yokohama and Vandercook 1967
Pulp	0.6	30% β-cryptoxanthin 22% phytofluene	Yokohama and Vandercook 1967
Eureka Fr.			
Pulp	0.3	56% β-cryptoxanthin 43% ζ-carotene	Fanciullino *et al.* 2006
Lisbon			
Peel	90.0	86% phytoene	Kato *et al.* 2004
Pulp	traces		Kato *et al.* 2004
Meyer			
Pulp	1.3	68% β-cryptoxanthin 15% β-carotene	Fanciullino *et al.* 2006
Volkamer			
Pulp	6.5	38% β-cryptoxanthin 15% violaxanthin	Fanciullino *et al.* 2006

Table 4 Carotenoid content and composition in the peel and pulp of mature fruits of selected pummelo (*Citrus grandis* Osbeck) cultivars.

Cultivar	µg/g FW	Carotenoids	Reference
Chandler			
Peel	19.3	32% lutein 22% violaxanthin	Gross 1987
Pulp	13.2	90% lycopene 5% β-carotene	Gross 1987
Chuzhou Early Red			
Peel	4.8	31% phytoene 25% phytofluene	Xu *et al.* 2006a
Pulp	5.3	51% lycopene 20% β-carotene	Xu *et al.* 2006a
Goliath			
Peel	5.0	67% phytofluene	Gross and Timberg 1983
Pulp	0.4	23% lutein 19% phytofluene 12% β-carotene	Gross and Timberg 1983
Yuhuan			
Pulp	traces	35% phytoene 35% lutein	Xu *et al.* 2006a

and red-fleshed (Chandler and Chuzhou Early Red) varieties. Flavedo and pulp of most white pummelo fruits accumulated almost exclusively linear (phytoene, phytofluene and lycopene) and bicyclic (β-carotene) carotenes, explaining the low coloration of these tissues. Carotenoid content in the flavedo ranged between 5 and 15 µg/g FW, while in the pulp it is almost zero. Two main features distinguish red pummelo fruit: the presence of lycopene, even at low concentrations but conferring the characteristic color, and that the carotenoid content is similar in both peel and pulp tissues (**Table 4**).

Contrary to the pattern of carotenoid accumulation during maturation of colored Citrus fruits, in pummelo the maximum carotenoid content was detected in green fruits and decreased thereafter. In the pulp, carotenoid content is very low and constant throughout the ripening process (Gross and Timberg 1983; Matsumoto *et al.* 2007).

Grapefruits

Grapefruit (*Citrus paradisi* Macf.) is considered a natural hybrid between a female pummelo and sweet orange. Attending to fruit color, grapefruits may be classified in two main groups: 1) fruits with pale to yellow pulp, and 2) fruits of pigmented pulp. In the latter, pulp color varies greatly from slight pinky to deep red (**Fig. 5**). The peel may also display red colorations although normally of less intensity than the pulp. Grapefruit is the *Citrus* species with the largest number of red mutants (Xu *et al.* 2006a).

Studies of carotenoids in grapefruits have been mainly focussed in mature fruits (**Table 5**) and evolution of carotenoid content and composition during ripening has received less attention. The seedless Marsh is the most cultivated variety in the world and the best characterized. During the transition from chloroplast to chromoplast, the content of total carotenoids in the flavedo decreased but increased the concentration of phytoene and phytofluene. In the pulp, phytoene and phytofluene also increased and may reach up to 75% of total carotenoids at the ripen stage (Yokoyama and White 1967; Banet *et al.* 1981; Xu *et al.* 2006a). Low levels of ζ- and β-carotene, and some xanthophylls, as 9-*cis*-violaxathin and auroxanthin, have been also detected in Marsh fruit (Banet *et al.* 1981; Fanciullino *et al.* 2006; Xu *et al.* 2006a).

Early investigations on carotenoids in red-grapefruits documented the presence of lycopene and β-carotene as the pigments responsible for the coloration of these fruits (Matlack 1935; Khan and Mackinney 1953). A correlation between lycopene content and the intensity of red coloration in pulp was established (Matlack 1935; Ting and Deszyck 1958). In addition to lycopene and β-carotene, which may account from 70 to 95% of total carotenoids, phytoene, phytofluene and ζ-carotene have also been detected in the pulp of different red grapefruit varieties (Khan and Mackinney 1953; Curl and Bailey 1957b; Gross 1987; Fanciullino *et al.* 2006; Xu *et al.* 2006b; Matsumoto *et al.* 2007). In the flavedo predominates colorless carotenes (64-95% of total), with reduced levels of xanthophylls, mainly violaxanthin and zeaxanthin (Gross 1987; Xu *et al.* 2006a). It is noteworthy that in some red grapefruits, fruit tissues that usually do not accumulate carotenoids, as albedo, are also coloured and contain lycopene and β-carotene (Xu *et al.* 2006b). As indicated in pummello, carotenoid content in flavedo and pulp of red grapefruits is very similar (Gross 1987; Xu *et al.* 2006a).

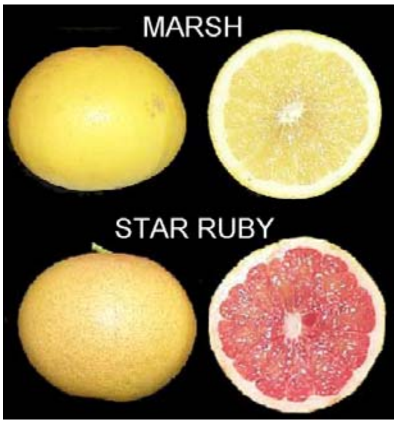

Fig. 5 External and internal appearance of mature white and red grapefruits (*Citrus paradisi* Macf.).

Table 5 Carotenoid content and composition in the peel and pulp of mature fruits of selected different grapefruit (*Citrus paradisi* Macf.) cultivars.

Cultivar	µg/g FW	Carotenoids	Reference
Marsh			
Peel	0.9-1.5	30-50% phytoene 5-24% phytofluene 5-16% violaxanthin	Yokoyama and White 1967; Xu *et al.* 2006a
Pulp	traces-2.0	17-63% phytoene 0-15% phytofluene 0-18% zeaxanthin	Banet *et al.* 1981; Fanciullino *et al.* 2006; Xu *et al.* 2006a
Ray Ruby			
Pulp	9.4	73% lycopene 12% β-carotene	Fanciullino *et al.* 2006
Ruby Red			
Peel	7.5-10.4	40-47% phytoene 14-17% phytofluene 1-24% violaxanthin	Curl and Bailey 1957; Xu *et al.* 2006a
Pulp	1.1-8.2	27-43% β-carotene 16-22% phytoene 2-40% lycopene	Curl and Bailey 1957; Xu *et al.* 2006a
Star Ruby			
Peel	62.3	47% phytoene 19% lycopene	Xu *et al.* 2006a
Pulp	17.0-53.0	48-57% lycopene 13-16% β-carotene 20% phytoene	Fanciullino *et al.* 2006; Xu *et al.* 2006

THE PATHWAY OF CAROTENOID BIOSYNTHESIS

The pathway of carotenoid biosynthesis in higher plants is now well characterized (**Fig. 6**). Carotenoids are synthesized in plastids (chloroplasts or chromoplasts), from nuclear-encoded enzymes. Carotenoids are derived by consecutive condensations of the five carbon precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In higher plants, these precursors are synthesized by two independent metabolic routes; the mevalonate (MVA) pathway which occurs in the cytoplasm and the more recently characterized methyl-D-erythritol 4-phosphate (MEP) pathway, taking place in the plastids. In light-grown plants, carotenoids are produced from MEP-derived precursors and the MVA pathway contributes to carotenoid biosynthesis in other instances, such as in etiolated tissues (Rodríguez-Concepción and Boronat 2002; Rodríguez-Concepción *et al.* 2004). The first reaction of the MEP pathway is the production of deoxyxylulose 5-phosphate (DXP), catalyzed by DXP synthase (DXS) using pyruvate and glyceraldehyde 3-phosphate as substrates. Intramolecular rearrangement and subsequent reduction of DXP by the enzyme DXP reductoisomerase yields MEP, considered the first committed precursor of the pathway. MEP is converted to IPP and DMAPP via different intermediates. Geranylgeranyl pyrophosphate (GGPP), a C_{20} molecule resulting by condensation of IPP, is the substrate and the entry point for carotenoid biosynthesis (Fraser and Bramley 2004; Bouvier *et al.* 2005).

The first committed step of carotenoid biosynthesis is catalyzed by the enzyme phytoene synthase (PSY), which converts two molecules of GGPP into phytoene via the intermediate prephytoene pyrophosphate. The colorless phytoene (C_{40}) undergoes four desaturation reactions catalysed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), thereby lengthening the carbon doubled-bonded series and yielding the orange prolycopene. These desaturations are connected to the photosynthetic electron transport

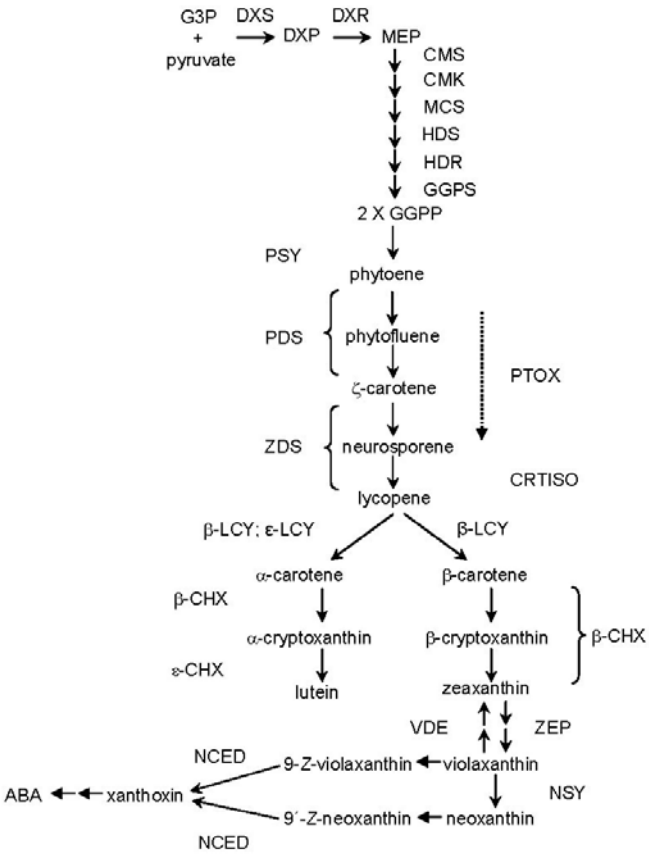

Fig. 6 Schematic diagram of the carotenoid biosynthesis pathway in plants. ABA, abscisic acid; β-CHX, β-carotene hydroxylase; ε-CHX, ε-carotene hydroxylase; CMK, 4-diphosphocytidyl-methylerythritol kinase; CMS, 4-diphosphocytidyl-methylerythritol synthase; CRTISO, carotene isomerase; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate-synthase; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; G-3-P, D-glyceraldehyde 3-phosphate; HDR; hydroxymethylbutenyl 4-diphosphate reductase; HDS, hydroxymethylbutenyl 4-diphosphate synthase; β-LCY, lycopene β-cyclase; ε-LCY, lycopene ε-cyclase; MCS, methylerythritol 2,4-cyclodiphosphate synthase; MEP, 2-C-methyl-D-erythritol 4-phosphate; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NSY, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene syn-thase; PTOX, plastid terminal oxidase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase.

chain via plastoquinone. In addition, the activity of a recently characterized ζ-carotene isomerase (Z-ISO) catalyzes the *cis*- to *trans*- conversion of the product of PDS (Li *et al.* 2007). Prolycopene is subsequently isomerised to the *trans*-form by carotene *cis-trans*-isomerase (CRTISO). Photoisomerization can substitute the CRTISO activity in chloroplasts (Park *et al.* 2002; Isaacson *et al.* 2004). The subsequent cyclization of the linear lycopene to yield β-carotene and α-carotene is the first branching point in the pathway. The formation of β-carotene is catalysed by a single activity, lycopene β-cyclase (β-LCY), whereas in the case of α-carotene two different enzymes, β-LCY and lycopene **ε**-cyclase (**ε**-LCY), are involved. α- and β-carotene are sequentially hydroxylated at the ring by ε- and β-carotene hydroxylases (ε-CHX and β-CHX) yielding oxygenated xanthophylls such as lutein (β,ε-xanthophyll derived from α-carotene) and β-cryptoxanthin and zeaxanthin (β,β-xanthophylls derived from β-carotene). The hydroxylated β-ionone rings of zeaxanthin can be modified by zeaxanthin epoxidase (ZEP) to form violaxanthin via anteraxanthin. De-epoxidation of violaxanthin to form zeaxanthin is catalyzed by violaxanthin de-epoxidase (VDE), which is activated by light. This reversible reaction is known as the “xanthophyll cycle”, which is involved in the dissipation of excess of light energy and hence plays a central role in light stress management. One epoxy ring of violaxanthin can be rearranged to form an allenic bond by neoxanthin synthase (NSY) to generate neoxanthin. All carotenoid biosynthetic enzymes are nuclear encoded, translated as precursors and imported post-translationally into the plastids (Sandmann 1994; Fraser and Bramley 2004; Bouvier *et al.* 2005).

Carotenoid biosynthetic genes in citrus fruits

Isolation and characterization of carotenoid biosynthetic genes in citrus have been recently reported (Kato *et al.* 2004; Rodrigo *et al.* 2004). Most studies have correlated the evolution of carotenoids content during fruit ripening with the accumulation of transcript of carotenoids biosynthetic genes. Different patterns of gene expression have been established, suggesting a complex transcriptional coordination in the metabolic events driven carotenogenesis in citrus fruit.

Two *PSY* genes in the *Citrus* genome have been inferred (Ikoma *et al.* 2001; Kim *et al.* 2003; Fanciullino *et al.* 2007; Tao *et al.* 2007). However, *PSY* sequences so far isolated are 97-100% identical. PSY is imported into plastids and peripherally associated to the membrane (Inoue *et al.* 2006) and its catalytic activity has been tested (Kim *et al.* 2003). Expression of *PSY* during fruit maturation appears to be a key regulatory step, as might determinate the entry of metabolic precursors to the massive increase of carotenoids. *PSY* transcript accumulation is lower in the pulp than in the flavedo and is well related to carotenoid content (Kato *et al.* 2004; Rodrigo *et al.* 2004; Tao *et al.* 2007; Alquézar *et al.* 2008; Fanciullino *et al.* 2008). Accumulation of *PSY* transcript has been also detected in vegetative tissues (Ikoma *et al.* 2001; Rodrigo *et al.* 2004). In tomato, two *PSY* genes have been detected, *PSY-1* encodes a fruit ripening specific isoform, whilst *PSY-2* predominates in green tissues and was not expressed in ripening fruit (Bramley 2002).

In citrus *PDS* gene is believed to belong to a low-copy number gene family (Kita *et al.* 2001; Fanciullino *et al.* 2007). On the contrary, in tomato and pepper fruits *PDS* is a single copy gene (Fraser and Bramley 2004). Partial or full-length sequences of PDS had been isolated from *C. maxima*, *C. paradisi*, *C. sinensis*, *C. unshiu* and *C. limon*, sharing 98-100% of identity. *In vitro* assays indicated that two processed forms of PDS are mainly targeted to the soluble fraction of plastids, while a minor unprocessed form appears peripherally associated to the outer surface of the membrane (Inoue *et al.* 2006). In other plant species, PDS exist in two forms, one bounded to the membrane and other stroma-associated (Lopez *et al.* 2008). Expression of *PDS* gene during fruit maturation has been analyzed in different orange cultivars (Kato *et al.* 2004; Rodrigo *et al.* 2004; Alquézar *et al.* 2008; Fanciullino *et al.* 2008), Satsuma mandarin (Kita *et al.* 2001; Kato *et al.* 2004) and Lisbon lemon (Kato *et al.* 2004). The abundance of *PDS* mRNA is low in the peel of green fruits, slightly increased during maturation to remain constant in the peel or to increase continuously in the pulp of mature fruit. In both tissues, flavedo and pulp, accumulation of *PDS* mRNAs was lower than that of *PSY*.

ζ-carotene desaturase (*ZDS*) is thought to be in at least three copies in the citrus genome, with polymorphism in at least two of them (Fanciullino *et al.* 2007). Results obtained in the yellow orange Pinalate mutant, suggest that may exist two ZDS isoforms, one chloroplast- and one chromoplast-specific (Rodrigo *et al.* 2003). *ZDS* sequences from *C. maxima*, *C. paradisi*, *C. sinensis*, *C. unshiu*, *C. clementina* and *C. limon* show an overall identity from 97 to 100%. *ZDS* transcripts were detected in young and mature leaves (Rodrigo *et al.* 2004). In flavedo and pulp of orange, mandarin and lemon fruits, *ZDS* mRNAs increased progressively with fruit maturation (Kato *et al.* 2004; Rodrigo *et al.* 2004; Alquézar *et al.* 2008; Fanciullino *et al.* 2008).

Beside desaturases, other activities such as PTOX, or the isomerases CRTISO or Z-ISO, are necessary for the correct desaturation of phytoene to lycopene. In tomato or maize, a defective activity of any of these enzymes leads to alterations in carotenoid accumulation (Josse *et al.* 2000; Isaacson *et al.* 2002; Li *et al.* 2007). The expression of *PTOX* and *CRTISO* citrus homologous is low in green fruits. During fruit maturation, expression of *PTOX* increased in parallel with that of *PDS* and *ZDS* (Rodrigo *et al.* 2004; Alquézar *et al.* 2008), while that of *CRTISO* remained constant or even decreased (Kato *et al.* 2004).

Two genes coding LCY had been identified in citrus fruits: ε-*LCY* and β-*LCY*. By RFLP analysis it has been established that β-*LCY* is present in a single locus in the citrus genome, while ε-*LCY* is present at one locus in *C. maxima* and *C. medica* genomes but at two loci in mandarin and other citrus genomes (Fanciullino *et al.* 2007). The nucleotide sequences of citrus β-*LCY* are at least 98% identical and code enzymes 98-100% identical. ε-*LCY* and β-*LCY* genes code proteins 55-56% homologous (38-39% identical). The ability of both enzymes to cycle lycopene has been recently confirmed (Inoue *et al.* 2006). β-LCY forms two β-rings in both extremes of the lineal molecule of lycopene, yielding β-carotene, and ε-LCY introduce a single ε-ring yielding δ-carotene. In ripening citrus fruits, the expression of ε-*LCY* is down-regulated, while that of β-*LCY* is constitutive or increased slightly (Kato *et al.* 2004; Rodrigo *et al.* 2004; Alquézar *et al.* 2008; Fanciullino *et al.* 2008). The expression of both *LCY* genes are also detected in leaves (Rodrigo *et al.* 2004). Recently, a citrus gene with high homology to a chromoplast-specific β-*LCY* from tomato (Ronen 2000) has been isolated. By functional assays in *E. coli* the ability of the encoded protein to convert lycopene into β-carotene has been confirmed. The expression of this new β-*LCY* is strongly induced during chloro- to chromoplast transformation, suggesting a role in the massive accumulation of β,β-xanthophylls during fruit maturation (Alquézar 2008).

In the genome of citrus, β-*CHX* gene is thought to be monolocus with two or three alleles (Kim *et al.* 2001; Fanciullino *et al.* 2007). In other fruits, as tomato, a green-specific and a flower/fruit-specific β-*CHX* genes have been detected (Hirschberg 2001). Citrus β-CHX is an integral membrane protein but whether it is directly associated or participating in a multienzymatic complex remains to be determined (Inoue *et al.* 2006). Expression of β-*CHX* gene increased substantially during maturation of orange, mandarin and lemon fruits (Kato *et al.* 2004; Rodrigo *et al.* 2004; Alquézar *et al.* 2008; Fanciullino *et al.* 2008). In addition, transcripts accumulation was hardly detectable in leaves and tissues from green fruits. Recently, it has been described the expression of a putative citrus ε-*CHX*, which is down-regulated during ripening of orange fruit (Alquézar *et al.* 2008).

ZEP gene is supposed to be present at two different loci in Citrus genome, both showing polymorphism (Fanciullino et al. 2007). cDNAs clones of *ZEP* highly homologues have been obtained from C. *unshiu*, *C. maxima*, *C. sinensis* and *C. limon*. The expression of this gene increased during maturation of orange, mandarin and lemon fruits (Kato *et al.* 2004; Rodrigo *et al.* 2004; Fanciullino *et al.* 2008; Alquézar *et al.* 2008).

Regulation of carotenoid biosynthesis in citrus fruits

Understanding how the expression of the different genes of carotenoid biosynthesis are coordinated during fruit maturation, and how the differences and singularities in peel and pulp color between fruits of the different cultivars and mutants are linked to altered mechanism of gene expression are primary goals for *Citrus* breeding. Application of modern molecular biology and genetic engineering strategies, and more recently, high-throughput genomic technologies, are now helping us to elucidate these processes and how are regulated.

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-*cis*-violaxanthin the main carotenoid in the peel and pulp of mature orange-colored fruits, as oranges and mandarins. The massive increase in total carotenoids and β,β-xanthophylls occurring during the transition from chloroplast to chromoplast appears to be coordinated by concomitant changes in gene expression. First, a substantial induction of phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), and β-carotene hydroxylase (*β-CHX*) gene expression (Kato *et al.* 2004; Rodrigo *et al.* 2004; Alquézar *et al.* 2008). Second, the shift from the ε,β-branch to the β,β-branch of the pathway is paralleled with a down regulation of the lycopene ε-cyclase (*ε-LCY*) gene expression. And third, in mandarin fruit, an up-regulation of lycopene β-cyclase (*β-LCY*) gene expression is also taking place (Kato *et al.* 2004). In the pulp of oranges and mandarins fruits, total carotenoid content is usually lower than in the peel (**Tables 1-5**), in accordance with a reduced expression of most carotenoid biosynthetic genes (Kato *et al.* 2004; Alquézar *et al.* 2008). These results suggest that a complex coordination among different steps of the pathway may operate to determine the final color and carotenoid compliment in citrus fruits. Evidences suggest that differences may exist among the different *Citrus* species and varieties, but *PSY* and *β-CHX* play central roles in rate of fruit coloration and in carotenoid accumulation.

Studies of carotenoid gene expression in fruits of different *Citrus* species indicate that carotenoid accumulation and composition during maturation is highly regulated by the coordinated transcriptional activation of carotenoid biosynthetic genes, as it has been demonstrated in other fruits. These evidences also stress that the balance or relative expression profile between the expression of upstream and downstream genes of the pathway are also important factors for the diversity in fruit color and carotenoids content. For example, differences in the expression balance between carotene (*PSY*, *PDS*, *ZDS* and *β-LCY*) and xanthophyll biosynthetic genes (*β-CHX* and *ZEP*) have been related to β-cryptoxanthin accumulation. The relative balance between the expression levels of carotene to xanthophyll genes was higher in Satsuma mandarins than in Valencia orange, which also accumulated higher concentration of β-cryptoxanthin (Kato *et al.* 2004). A similar relationship has been also found in ethylene-treated Satsuma fruits (Fujii *et al.* 2007). Other example is the massive accumulation of phytoene in flavedo of Lisbon lemon, which was correlated with a decrease in the transcript level of *PDS* (Kato *et al.* 2004). However, in other instances the phenotypic differences in fruit color could not be explained by transcriptional changes of carotenoid biosynthetic genes, suggesting that other still unknown regulatory mechanisms may be also involved (Fanciullino *et al.* 2008). Transcriptional, post-transcriptional and translational regulation of gene expression, catalytic activity and membrane-associated complexes have been postulated to be mechanism of regulation of carotenoid biosynthesis in other plants (Cunningham and Gantt 1998; Bramley 2002).

Recently, it has been reported that alterations in the expression of genes of the MEP pathway may be related to changes in fruit color and total carotenoid content. Changes in the levels of *DXS* have been reported in fruits of four orange varieties (Fanciullino *et al.* 2008). Furthermore, accumulation of lycopene in the pulp of the red-fleshed-mutant Cara Cara has been related with an enhanced expression of MEP genes, in particular *HDS*. In the mutant, *β-LCY* was also up-regulated, suggesting a positive feedback regulatory mechanism of carotenoid biosynthesis (Alquézar *et al.* 2008). In other plants, biotechnological up-regulation of MEP-related genes resulted in enhanced accumulation of carotenoids, indicating that precursor supply may be an additional control point of carotenoid biosynthesis (Estevez *et al.* 2001; Mahmoud and Croteau 2001; Botella-Pavía *et al.* 2004; Carretero-Paulet *et al.* 2006).

Carotenoid catabolism

The oxidative cleavage of carotenoids leads to the production of apocarotenoids. Enzymes catalyzing these reactions are generically recognized as carotenoid cleavage dioxygenases (CCDs). Three *CCDs* genes have been identified from citrus fruits, although catalytic activity of the encoded enzyme has only been confirmed in one of them (Kato *et al.* 2006; Agustí *et al.* 2007). Some citrus apocarotenoids, as β-citraurin, are genus specific (**Fig. 2**) and greatly contributes to fruit color. β-citraurin is believed to be a degradation product of either zeaxanthin or β-cryptoxanthin (Yokoyama and White 1966; Farin *et al.* 1983; Rodrigo *et al.* 2004; Rodrigo and Zacarías 2007), however, the enzyme/s involved in its biosynthesis is still unknown. The plant hormone abscisic acid (ABA) is also an apocarotenoid derivate. The key step in ABA biosynthesis is the oxidative cleavage of *cis*-epoxycarotenoids, by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), a subfamily of CCDs. The two *NCED* genes characterized in citrus fruits showed differential patterns of expression during normal fruit development and under stress conditions (Kato *et al.* 2006; Rodrigo *et al.* 2006; Agustí *et al.* 2007).

FACTORS AFFECTING CAROTENOID CONTENT AND COMPOSITION

It is generally accepted that the chloro- to chromoplast transformation is a major physiological event affected by environmental, nutritional and hormonal factors. This process comprises the progressive degradation of chlorophylls that is comparable with the senescence of vegetative tissues, and also the gradual accumulation of carotenoids (Goldschmidt 1988). Biogenesis of chromoplasts is then a unique metabolic process of agronomical and commercial relevance in citrus and that, under particular circumstances, may be reversible. Besides the genetic nature of citrus fruit to accumulate diversity of carotenoids in a specific pattern during ripening, there are a series of endogenous and exogenous factors that deeply influence carotenoid content and composition. High accumulation of lycopene in grapefruits growing under warm climatic conditions or the inability to develop full color of tropical-grown fruits, are two classical examples of the climatic influence in citrus fruit color. Following is a summary of the main effects of selected endogenous and exogenous factors on fruit color and carotenoid content and composition.

Hormones

The influence of phytohormones in the color of citrus fruits is long time recognized and has been the basis of selected agronomical treatments aimed to manipulated the rate of fruit coloration. Citrus fruits are classified as non-climateric, but studies with inhibitors of ethylene action revealed that ethylene plays a role in citrus fruit ripening (Goldschmidt *et al.* 1993; McCollum 2007). Ethylene is being used to stimulated postharvest fruit coloration for most than a century (Grierson *et al.* 1986). This commercial treatment is of particular interest in fruits of early season cultivars, since the pulp reaches maturity and becomes edible when the peel is still green. Ethylene application is also used to uniform external coloration in fruit submitted to external markets (Saltveit 1999). The efficiency of ethylene enhancing external fruit color is higher in more mature fruit (Stewart and Wheaton 1972; Eilati *et al.* 1975; Rodrigo and Zacarías 2007).

The ethylene-induced peel coloration is due to a dual coordinative effect on the stimulation of chlorophyll degradation and on carotenoid synthesis. Ethylene has been demonstrated to induce chlorophyll loss and chlorophyllase gene expression (Stewart and Wheaton 1972; Trebish *et al.* 1993; Jacob-Wilk *et al.* 1999). However, the involvement of the hormone in natural fruit degreening is questionable, as chlorophyllase has been shown to be constitutively expressed during the whole process (Jacob-Wilk *et al.* 1999). Isolation of two chlorophyllase genes expressed with different pattern suggests the existence of two independent pathways of chlorophyll breakdown, an ethylene-independent and other ethylene-dependant (Fujii *et al.* 2007; Tadeo *et al.* 2008).

In addition to chlorophylls disappearance, ethylene stimulates carotenoid accumulation. Earlier studies reported than ethylene improves the external color of orange fruit by inducing accumulation of orange and red carotenoids, such as β-cryptoxanthin and β-citraurin (Stewart and Wheaton 1972). During ethylene-induced coloration, the profile of carotenoid accumulation in Shamouti and Navel oranges are similar to that observed during natural ripening. Ethylene-treated fruits accumulated higher content of phytoene, β-cryptoxanthin and β-citraurin than control fruits (Eilati *et al.* 1975; Rodrigo and Zacarías 2007). Ethylene also up-regulated the expression of *PSY*, *ZDS* and β-*CHX* genes, sustained o transiently increased the expression of *PDS*, *PTOX*, β-*LCY* and *ZEP*, and decreased the expression of ε-*LCY*. Taken together, the results demonstrated that ethylene reproduces and accelerates the biochemical and molecular changes related to carotenoid biosynthesis naturally occurring during ripening of citrus fruits (Rodrigo and Zacarías 2007). However, the involvement of ethylene in the regulation of these processes during natural fruit ripening still needs further confirmation.

In contrast to ethylene, gibberellic acid (GA) is long time recognized as a retardant of fruit degreening (Cooper and Henry, 1968). Pre-harvest GA applications before the onset of color break is a common practice in many countries to delay the rate of fruit degreening in several *Citrus* species (Garcia-Luis *et al.* 1986; Porat *et al.* 2001). Field application of GA to mature green Clemenules mandarin resulted in a reduction of chlorophyll depletion and the maintenance of ε,β-carotenoids in detriment of the flux through the β,β-branch (Alós *et al.* 2006). Postharvest-GA treatment had a marked effect preventing accumulation of phytoene and phytofluene. The treatment partially suppressed ethylene-induced carotenoid changes, as accumulation of linear carotenes and β-citraurin, the decrease in lutein and all-*E*-violaxanthin, but did not affect 9-*cis*-violaxanthin content. GA-application also counteracted the changes in carotenoid gene expression stimulated by ethylene (Rodrigo and Zacarías 2007). These results are consistent with observations obtained in field-grown Clementine mandarins in which gibberellins appears to operate via repression of the ethylene-induced color changes mediated by sugars (Iglesias *et al.* 2001).

The action of cytokinins delaying color changes in citrus fruits is controversial. In mature green oranges and mandarins, application of the synthetic cytokinin N^6-benzyladenine counteracted the ethylene-induced chlorophyll degradation (Eilati *et al.* 1975), but it did not retarded natural fruit degreening (Goldschmidt *et al.* 1977; Garcia-Luis *et al.* 1986). However, cytokinins also reduced carotenoid accumulation during maturation in a fashion similar to that of gibberellins. Thus, it appears that the action of both retardant hormones is different in the metabolic events leading to chlorophyll degradation and carotenoid accumulation during fruit maturation

During natural maturation, the peel of citrus fruits accumulates large amounts of abscisic acid (Lafuente *et al.* 1997; Rodrigo *et al.* 2006; Agustí *et al.* 2007), which is derived from the oxidative cleavage of 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin (Rodrigo *et al.* 2006). Evidences suggested that ABA may be involved in chromoplast development and/or the rate of fruit coloration in citrus fruit (Harris and Dugger 1986; Kato *et al.* 2006). In support of the hypothesis is the observation that the rate of peel degreening is delayed in an ABA-deficient orange mutant (Rodrigo *et al.* 2003). Ethylene-induced fruit degreening is also impaired in that mutant, suggesting that ABA may play a role in the sensitivity of flavedo to ethylene (Alférez and Zacarías 1999).

Environmental factors

It is well known that climactic factors play a pivotal role in development of fruit color and hence carotenoid content and composition. Data are fragmentary and disperse, and additional research is required to understand the influence of environmental conditions on carotenoid biosynthesis at the biochemical and molecular levels.

a) Temperature

Generally, optimal temperature for carotenogenesis in plants is relatively low. Citrus fruits have special temperature requirement not encountered in other fruits, as conversion of chloroplast into chromoplasts is induced by rather low temperatures (Gross 1987). A study conducted in Florida with Hamlin, Parson Brown and Pineapple oranges found that color break occurs at about 13°C (Stearns and Young 1942). Young and Erickson (1961) confirmed these findings and demonstrated that rate of fruit coloration depends on the reduction and on the length of time below a threshold temperature. A temperature day/night regime of 20/7°C induced bright orange fruits in Valencia. Higher day/night temperatures, however, resulted in less intense color. It was then suggested that night temperatures below 20°C stimulated xanthophyll accumulation. Meredith and Young (1969) working with Redblush grapefruit and Ruby blood sweet orange, indicated that low day/nigh temperature (16/5°C) are required for carotenoid formation, but higher day/night temperatures (35/30°C) were necessary for lycopene formation. It is important to stress that temperature higher than 35°C also inhibited lycopene biosynthesis (Meredith and Young 1971). As a general rule, high temperatures produced fruit with low carotenoid content and high chlorophyll levels (Stearns and Young 1942; Young and Erickson 1961; Agusti 1999). Moreover, the main reddish pigment of the peel, β-citraurin, is temperature sensitive, and its synthesis decrease at temperatures above 30°C (Stewart and Leuenberger 1976). More recently, a postharvest simulation of low orchard temperature increased carotenoid content and improved rind color of Nules Clementine mandarin (Barry and van Wyk 2006). All these observations explained the inability of citrus fruits grown in the tropics to develop their characteristic color, especially orange and mandarins, which acquire a greenish-pale peel coloration. However, under these environmental conditions the pulp develop the typical orange color, indicating that peel and pulp behave as separate organs with differential requirements and regulatory mechanism, at least for carotenoid biosynthesis (Tadeo *et al.* 2008).

The effect of whole tree temperature on carotenoid content may be mediated by the hormonal status of the root. Sonnen *et al.* (1979) analyzed the effect of two root temperatures (14 and 30°C) on carotenoid content in the peel of Satsuma mandarin. At low temperature, fruit degreening was faster and total carotenoids were higher and with a more complex pattern. It was then suggested that 12-14°C are the optimal temperature for β-cryptoxanthin, β-citraurin and violaxanthin synthesis. This requirement of low soil temperature for color development might be connected with the reduction of the color-retardant hormones gibberellins and cytokinins on the roots and hence for fruit supply (Eilati *et al.* 1969).

b) Light

Light is also an important factor for carotenoid synthesis, although it appears not to be an essential requirement, since the pulp may also accumulate diversity of carotenoids. Evidences accumulated over the years in fruits of oranges and mandarins cultivated in different countries, indicated that fruits located into the tree canopy developed low peel color than those located in the outer canopy, under a high light intensity (Sites and Reitz 1949; Iwagaki and Kudo 1977; Iwagaki 1981; Uchida *et al.* 1985; Casas and Mallent 1988). Uchida *et al.* (1985) reported that a reduction of light intensity to 1.5% of normal, by bagging the fruits with black-colored polyethylene-film, substantially reduced peel coloring. It has been also reported that citrus fruits exposed to illuminations lower than 40% of the usual, showed lower coloration and that the reduction in coloration was proportional to the reduction of irradiation (Iwagaki and Kudo 1977). Lewis and Coggins (1964) established that fruit exposed to 5% of the usual illumination had 42% less carotenoids compared to fruit exposed to normal illumination. These data suggested that citrus peel coloration is highly influenced by the quantity and the quality of the light received by the fruit.

Other results indicate that carotenoid content in the peel of Navel oranges located outer or inner the tree canopy was similar, but the disappearance of chlorophylls occurred earlier in the peel of the fruits from the outer canopy (Casas and Mallent 1988). Recently, it has been reported that shaded Hongshigan orange developed lower coloration that unshaded fruits and this effect was due to a rapid reduction of chlorophyll content and lower carotenoid content. After exposure to light, carotenoid content and β-cryptoxanthin were again recovered (Tao *et al.* 2005). It appears then that light is an environmental signal essential for carotenoid synthesis in citrus fruit during certain stage of fruit development.

The influence of light on fruit color and carotenoid content seem not to be the same among the different citrus cultivars, as opposite effects has been observed in orange and mandarins respect to red grapefruits. Meredith and Young (1969) reported a positive influence of reducing light intensity on Redblush grapefruit coloration. Fruits exposed to reduced light intensity accumulated higher lycopene content than control fruits. We have also observed that Star Ruby grapefruits located in the inner canopy presented a more intense red color that those of the outer canopy. Shaded external fruit resulted in an increased color and lycopene content. Analysis of carotenoid gene expression did not reveal significant changes between inner- and outer-grown fruit, suggesting that other factors are involved in the effect of light on carotenoid content (Alquézar 2008).

Nutritional factors

Large body of evidences demonstrated that nutrients play pivotal roles in citrus fruit coloration (Agustí 1999). Fertilization with high doses of nitrogen is long time recognized to delay degreening of citrus fruits (reviewed by Casas and Mallent 1988). Studies of dose-response in calamondin revealed that low nitrogen fertilization increased carotenoid content whereas it was reduced at higher concentrations (Gross *et al.* 1979). Nitrogen also affected internal fruit quality of Hamlin, Pinneaple and Valencia orange (Koo and Reese 1977). It is then well established that nitrogen repressed chloroplast to chromoplast transformation in the flavedo of citrus fruits, and thus its depletion is a requirement for color change (Iglesias *et al.* 2001). Alos *et al.* (2006) have observed that high level of nitrogen in Clemenules mandarin delayed color changes and chlorophyll disappearance. Carotenoid content was also reduced and their profile was the typical of green tissues, with high content of *trans*-violaxanthin, neoxanthin, lutein and α-carotene, and lower levels of β-cryptoxanthin. These biochemical changes were concomitant with a delay in the expression of maturation-related carotenoid genes.

Rootstock is also an important factor for fruit quality and fruit color, and at least part of that influence may be related to an indirect effect on nutrients. Thus, scions on vigorous rootstocks have a higher hydraulic conductivity, allowing more mineral nutrients (e.g. nitrogen) to be transported to the leaves and fruits, and then the rate of ripening is delayed. Additional influence of the root-synthesized gibberellins or cytokinins can not be ruled out. The influence of irrigation on the content of carotenoids of the fruits may be also related to the nutrient balance. Valencia orange fruits from well irrigated trees accumulated more carotenoids than those under low irrigation (Koo and Reese 1977). Trickled irrigation of Valencia orange and Redblush grapefruit trees resulted in fruits with less carotenoids in the peel than that from flood irrigated trees. This effect has been associated with a better nitrogen uptake by the roots from trickle-irrigated tress. Contrary, lycopene content increased in Redblush grapefruit fruits from trickle irrigated tress (Huff *et al.* 1981).

Besides nitrogen, sugars are major effectors of maturation in *Citrus*. Results obtained using *in vitro* culture of isolated peel segments of citrus fruits demonstrated that accumulation of sugars stimulated peel degreening (Huff 1983, 1984). This effect was reversible, as peel re-greening was induced by depletion of sugar content. Further confirmation of these results was obtained by whole-tree experiments, in which it was concluded that conversion from chloroplast to chromoplast required as a first step reduction of nitrogen and subsequently is stimulated by sugars. This nutrient balance is connected with hormonal signals, as sugar-induced color changes are mediated by ethylene, and gibberellins may be repressors of the ethylene-sugar interaction (Iglesias *et al.* 2001).

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