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Stay-Green Phenotype Slows the Carotenogenic Process in *Capsicum annuum* (L.) Fruits

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Stay-green mutants have been very useful for elucidating the chlorophyll catabolism pathway in higher plants. In the present study the possible relationship between the retention/catabolism of chlorophylls and the carotenogenic process taking place in ripening Capsicum annuum (L.) fruits has been investigated. Phytylated, dephytylated and oxidized chlorophyll derivatives, and total and individual carotenoids were analyzed over the whole ripening period. In general terms, the biosynthesis of carotenoid pigments taking place during the ripening of *C. annuum* fruits is identical in both red and stay-green lines, so that the carotenogenic process is independent of the retention of chlorophylls. However, it has been found that the carotenogenesis is slowed in the stay-green lines. Therefore, although the catabolism of chlorophylls and biosynthesis of carotenoids seem to be separate processes, the fact that they are taking place in the chloroplast/chromoplast suggests that some kind of interaction between the two processes may occur at different levels. Plastids corresponding to the wild genotype (red color fruit phenotype) show high plastoglobuli density and thylakoids are almost absent, whereas in the case of stay-green phenotype, thylakoids and plastoglobuli coexist in the same plastid (chlorochromoplasts). The role of carotenoid pigments on the physiological mechanism for protecting the preserved thylakoid structures is discussed.

KEYWORDS: ***Capsicum annuum*; fruits; red; ripening; stay-green; carotenoids; chlorochromoplast; chlorophylls**

INTRODUCTION

During the ripening of *Capsicum annuum* L. fruits, *de novo* synthesis of carotenoid pigments occurs, some of them (capsanthin and capsorubin) being exclusive to this genus (*1*). This process is accompanied by a sharp decrease in chlorophylls as a consequence of the degeneration of chloroplasts into chromoplasts. The rapid disappearance of chlorophylls (chls) is accompanied by the disintegration of the intricate thylakoid membrane system and the deposition of carotenoids in less elaborate membranous structures such as globules and crystals within the chromoplast envelope (*2*). Recently, Ytterberg et al. (*3*) analyzed the plastoglobule proteome of red peppers, identifying up to 28 proteins, including four enzymes involved in the carotenoid biosynthetic pathway, as well as fibrilins, aldolases, glucosidases, and others. In carotenogenic fruit chloroplasts rapidly lose their photosynthetic capacity during differentiation into chromoplasts, and along with this process is the decline of the levels of proteins involved in photosynthetic reactions (*4*).

The chlorophyll catabolic pathway denominated PaO (pheophorbide *a* oxygenase) is initiated by two consecutive reactions governed by chlophyllase and Mg-dechelatase (or MCS: metal chelating substance; *5*), that catalyze the respective elimination of phytol and Mg^{2+} from the chlorophyllic molecule, eventually giving way to pheophorbide *a*. Subsequently, the porphyrin ring of pheophorbide *a* is oxygenolytically opened, producing noncolored chlorophyll catabolites (NCCs) (*6*). For this biochemical pathway, three genes have already been cloned and their enzymes characterized: chlorophyllase (*7*, *8*), RCC reductase (RCCR) (*9*), and PaO (*10*).

Stay-green is the general term given to a variant in plants in which senescence (visually perceived by the loss of chlorophylls) is delayed compared with a standard reference genotype (*11*). The so-called "cosmetic" stay-green genotypes are characterized by a total decline of the photosynthetic activity together with a partial retention of chlorophylls. In general, the protein complexes associated with chlorophylls are also retained in the senescent stay-green mutant at the same level than in the green fruits (*10*, *12*). In these stay-green mutants the PaO pathway is blocked.

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Table 1. Characterization of the Selected Lines of *Capsicum annuum* (L.) Fruits Analyzed with Respect to Phenotype

	superficial color									
	M variety		R variety			D variety		L Variety		
stage	Mr 1	Mn 3	Rr 1	Rn 1	Rn 2	Dr 6	Dn 3	Lr 2	dLr 7	Negral variety
harvesting	red	brown	red	brown	brown	red	brown	red	red	brown
overripe	red	red	red	red	red	red	red	red	red	brown

The situation is more complex in the carotenogenic fruits with stay-green phenotype, in which retention of chlorophyll in the degenerating chloroplast and *de novo* biosynthesis of carotenoids in the upcoming chloroplast coexist. A well-known example for this situation is the tomato (*Lycopersicum sculentum* Mill.) green flesh mutant (*gf*) (*13*). At metabolic level, it seems that the mutation responsible for that character avoids the actuation of the PaO enzyme, whether by an alteration in the accessibility or transport of components required for the thylakoid disassembly or by the absence of any other factor (*14*). As a result, granum structures (staked thylacoids) typically present in the chloroplast coexist with specific structures of the tomato chromoplast (*2*). At a molecular level, components of the photosynthetically active chloroplast are retained whereas in normal tomatoes these components eventually disappear as a result of ripening.

The main aim of the present work is to establish whether the carotenogenic process is affected in some way by the retention of chlorophylls in *C. annuum* fruits. For that purpose, a detailed analysis of the chlorophyll and carotenoid pigments composition has been carried out during ripening and overripening of fruits with red and stay-green phenotype.

MATERIALS AND METHODS

Plant Material. Fruits of the pepper (*Capsicum annuum* L.) of ten selected lines (Mr1, Mn3, Rr1, Rn1, Rn2, Lr2, Lr7, Dn3, Dr6, and Negral) were used for the present study. Plants were grown in open fields at the Centro de Investigaci'on y Desarrollo Alimentario (CIDA, La Alberca, Murcia, Spain). Five to ten fruits, depending on the size, at different ripening stages, were harvested every 15 days after anthesis during the growing and ripening period (75 days). Following harvesting, fruits were devoid of peduncles and seeds, cut into small pieces, lyophilized, and kept at −30 °C until analysis.

Pigment Extraction. Ten grams of fruit sample were extracted with 30 mL of acetone, repeating the operation until no more color was extracted. The combined extracts were poured into a separating funnel and treated with 100 mL of diethyl ether. To this mixture, 100 mL of 10% (w/v) sodium chloride was added; the ethereal phase was separated in two aliquots, each for chlorophyll and carotenoid analysis, respectively. The "chlorophyll fraction" was washed three times with 100 mL of 5% (w/v) anhydrous Na_2SO_4, filtered through a layer of anhydrous Na_2SO_4, and evaporated to dryness under vacuum. The residue was dissolved in 5 mL of acetone and kept at −20 °C until its analysis by HPLC. The "carotenoid fraction" was saponified with 40 mL of 20% (w/v) KOH−methanol during 1 h at room temperature. After addition of water, the pigments were subsequently extracted with diethyl ether, evaporated in a rotary evaporator, and taken up to 25 mL of acetone. A 1 mL aliquot of this solution was centrifuged at 12000 rpm and stored at −30 °C until analyzed. Losses occurring during the analytical procedure were monitored with use of *all-trans-β*-apo-8′-carotenal as internal standard. All analyses were carried out in triplicate.

HPLC Separation and Quantification of Chlorophylls and Carotenoids. This was carried out using an HP1100 Hewlett-Packard liquid chromatograph fitted with an HP 1100 automatic injector and Diode Array Detector. A stainless steel column C18 Spherisorb ODS-2 (25 × 0.46 cm, 5 μm) was used. Separation and quantification of the pigments was carried out following the method previously developed by the authors (*15*). Detection was performed simultaneously at 450 nm for carotenoids, 666 nm for chlorophyll *a* and derivatives, and 650 nm for chlorophyll *b* and derivatives. Response factors were calculated for each chlorophyll pigment by performing external standard calibration. In the case of carotenoid pigments, quantification was achieved by internal standard calibration using *all-trans-β*-apo-8′-carotenal. For the separation and quantification of zeaxanthin and lutein, the method of Juhler and Cox (1990) (*16*) was used.

Pigment Identification. This has been described in detail in previous publications: for chlorophyll catabolites, Mínguez-Mosquera et al. 1991 (17, *18*), for oxidized chlorophylls, Mínguez-Mosquera et al. 1993 (*19*), and for carotenoids, Mínguez-Mosquera and Hornero-Méndez, 1993 (*15*), and consist of the following: separation and isolation of the pigments by TLC and co-chromatography with standard pigments; observation of the pigment color on TLC plates under white, UV254 nm, and UV360 nm lights; acquisition of UV−visible spectra in different solvents and comparison with the values reported in the literature; chemical derivatization microscale tests.

Electron Microscopy. Fresh fruit samples were fixed with osmium tetroxide and glutaraldehyde using standard protocols for electron microscopy. Ultrathin sections were prepared using LKB-8800 ultratom and viewed on a transmission electron microscope (PHILIPS CM-200).

Statistical Analysis. CV was calculated using Statistica for Windows (version 5.1, StatSoft, Inc, Tulsa, OK) and always was less than 10%.

RESULTS AND DISCUSSION

Table 1 summarizes the characteristics of the *C. annuum* lines included in the present study. A distinction has been made between those lines with red color (red phenotype) at 45 days from those with brown color (stay-green phenotype) at the same ripening stage. Only in the case of fruit of the Negral variety, the stay-green phenotype is constant all throughout the entire ripening process and even when the fruit is overripe (75 days).

The chlorophyll fraction comprises mainly chlorophylls *a* and *b*, but depending on the genotype and ripening stage, some chlorophyll derivatives were detected, such as chlorophyllide *a*, pheophorbide *a*, and 13^2-OH-chlorophylls *a* and *b*. The initial values for total chlorophyll content (**Figure 1**) tended to be higher in the red lines (1100−1700 mg/kg dw) than in the stay-green lines (700−1100 mg/kg dw) with the exception of the Mn3 line reaching 1600 mg/kg dw. However, the results obtained after comparison of wild and stay-green lines for the same variety do not associate a higher or lower initial chlorophyll content with a specific phenotype.

After 45 days of the ripening onset, all the red lines had lost the chlorophyll pigments, whereas in the stay-green lines the chlorophyll concentration ranges from 550 to 900 mg/kg dw. Dephytylated chlorophyll compounds (chlorophyllide and pheophorbide) were not found in fruits corresponding to red lines (**Table 2**), suggesting that the rate of the chlorophyll catabolic reactions taking place in these types of pepper fruits prevents the accumulation of these colored catabolites. On the contrary, in the corresponding stay-green and Negral lines, accumulation of dephytylated catabolites was observed, in increasing concentration as the ripening progressed. Taking into consideration that the PaO pathway is the one operating for chlorophyll catabolism in pepper fruits (*20*), this accumulation

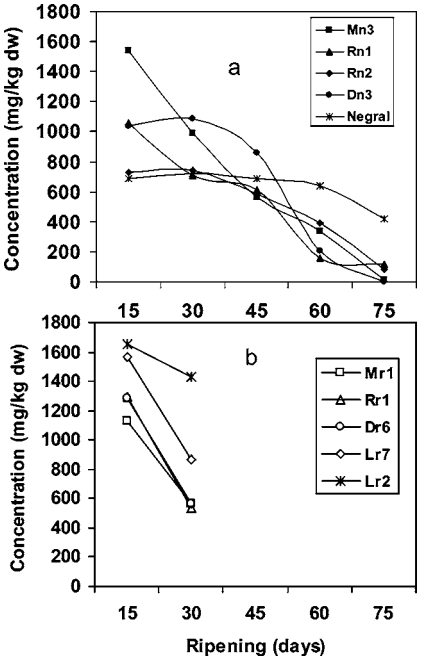

Figure 1. Total chlorophyll content in stay-green (a) and wild type (b) lines of *Capsicum annuum* L. fruits during the ripening process. Lines analyzed are described in **Table 1**. The total amount is the sum of the individual chlorophyll pigments identified and quantified by HPLC, as described in Materials and Methods. CV ≤ 10% in all cases.

Table 2. Changes of the Total Dephytylated Chlorophylls Fraction: Chlorophyllide and Phaeophorbide in Stay-Green Mutants of *Capsicum annuum* (L.) during the Ripening

	dephytylated chlorophylls (mg/kg dw)				
developing (days)	Mn 3	Rn 1	Rn 2	Dn 3	Negral
15	0.99	1.27	nd	nd	nd
30	2.92	nd[a]	1.54	nd	nd
45	nd	nd	nd	37.78	nd
60	40.28	28.24	22.50	52.60	nd
75	13.38	115.82	79.30	nd	12.48

[a] nd: nondetected. CV ≤ 10% in all cases.

in the stay-green lines is indicative of a mutation at the level of the PaO activity, the precedent enzymes, chlorophyllase and Mg-dechelatase, being active, as demonstrated by *in vivo* substrate modification (*18*). This same pattern has been described previously for other stay-green vegetables, such as senescent leaves of *Lolium temulentum* L. (*12*), in leaves of the mutant *lls1* of *Zea mays* L., and in leaves and fruits of the *gf* tomato mutant, in which there are an accumulation of dephytylated chlorophyll catabolites. In all of them the mutation responsible for the stay-green character has been associated to the PaO activity.

Regarding the carotenoid fraction, **Figure 2** represents the progress of the carotenogenic process during ripening for the red (**Figure 2A**) and stay-green phenotypes (**Figure 2B**). This pigment fraction for the initial growing and developing stages comprised the typical carotenoids present in the chloroplast of green vegetable tissues, namely β-carotene, lutein, violaxanthin, neoxanthin, and antheraxanthin. However, once the ripening process is triggered, and as a consequence of the overexpression of the carotenogenic pathway in *C. annuum* fruits, there is massive synthesis of carotenoids, some of which are already present in the green and unripe fruit, but others are formed *de novo*. The last fraction comprises yellow xanthophylls

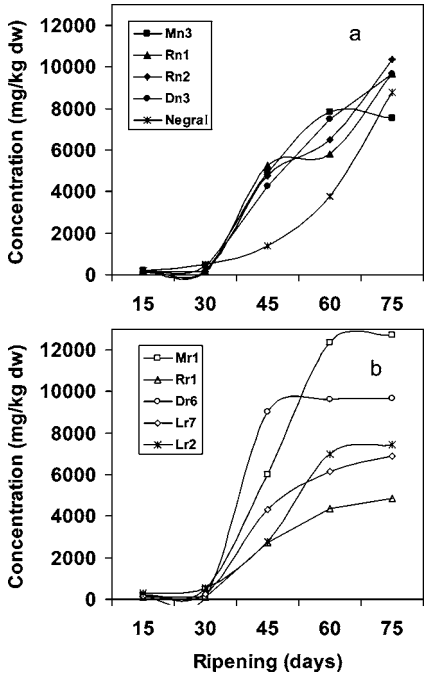

Figure 2. Total carotenoid content in stay-green (a) and wild type (b) lines of *Capsicum annuum* L. fruits during the ripening process. As in **Figure 1**, CV ≤ 10% in all cases.

(zeaxanthin, β-cryptoxanthin, and cucurbitaxanthin A) and the exclusive and characteristic red xanthophylls (capsanthin, capsorubin, and capsanthin 5,6-epoxide) (*21*).

Irrespective of the line phenotype, the total carotenoid content found during the growing and developing stages remains almost constant, with a slight decrease just before the onset of the carotenogenesis (*22*). The persistence of chlorophylls in the stay-green lines, together with the biosynthesis of red xanthophylls, is responsible for the brown color of the fruits.

Chlorophylls and carotenoids form part of the photosynthetic apparatus, either in reaction centers or in antenna complexes. Thus, changes in the composition of chloroplast pigments are a reflection of the modifications in the structure of the photosynthetic apparatus. Due to the fact that in stay-green lines from *Capsicum* both chromoplastic and chloroplastic structures coexist, it is in a way logical to think about the possibility of imbalance between the chlorophyll and carotenoid fractions.

Developed fruits have in general a chlorophyll/carotenoid (Chl/Car) ratio of about 3−4, decreasing as ripening starts and progresses, which is due to the chlorophyll catabolism taking place during senescence and ripening and usually before other pigments are synthesized (anthocyanins and carotenoids). The Chl/Car ratio may take values up to 8.0 in the unripe stage of some fruits such as "Granny Smith" apples (*27*) and green pepper (*28*). This fact has been observed in green fruits of some of the studied cultivars. The data obtained for these lines indicate that the chlorophyll-to-carotenoid balance is not affected by the retention of chlorophylls, with a nondefined tendency observed. In the stay-green lines, the slow chlorophyll catabolism contributes to a smoother decrease of the ratio values.

Although in both fruit types (red and stay-green) the maximum expression of the carotenogenesis occurs between 30 to 45 days of ripening, in the chlorophyll-retaining lines this biosynthesis almost linearly continues until the end of the controlled period, whereas in the fruits of the red lines the synthesis of carotenoid becomes stabilized from 45 days, coinciding with the chlorophyll disappearance. Consequently,

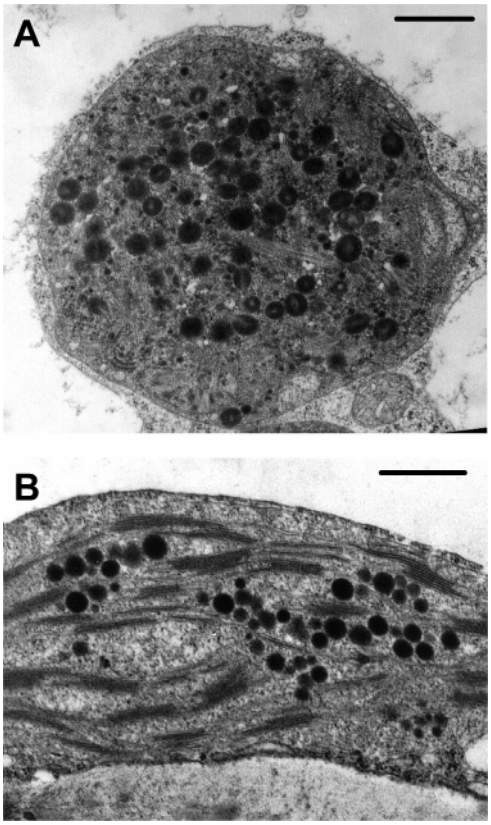

Figure 3. Ultrastructure of plastids from *Capsicum annuum* ripe fruits corresponding to red (A) and stay-green (B) lines of D variety at approximately 45 days of ripening. Bar = 0.5 μm.

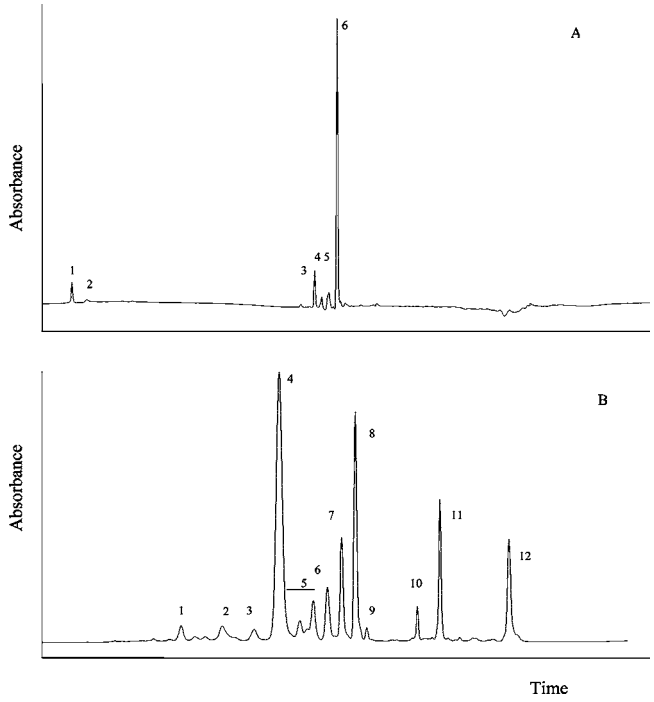

Figure 4. Reverse-phase HPLC chromatogram corresponding to sample Dn3 of *Capsicum annuu*m fruits at 45 days of ripening. (A) Chlorophyll extract, peak identities: 1, chlorophyllide *a*; 2, pheophorbide *a*; 3, OH chlorophyll *a*; 4, OH chlorophyll *a*′; 5, chlorophyll *b*; 6, chlorophyll *a*. (B) Saponified extract of carotenoid pigment peak identities: 1, capsorubin; 2, violaxanthin; 3, capsanthin-5,6-epoxide; 4, capsanthin; 5, *cis*-capsanthin; 6, antheraxanthin; 7, cucurbitaxanthin A; 8, zeaxanthin; 9, *cis*-zeaxanthin; 10, *all-trans*-β-apo-8′-carotenal (internal standard); 11, β-cryptoxanthin; 12, β-carotene.

in these fruits the persistence of chlorophylls extends the carotenogenic process. However, different patterns have been found for each variety, so that the stay-green lines for the R variety (Rn1 and Rn2) show a more pronounced carotenogenesis than the red line (Rr1); in the D variety both lines progress in a similar manner, while in the case of M variety, the red line has a higher carotenoid content at the end of ripening. In general terms, in the chlorophyll-retaining lines a slowing of the carotenogenic process could be assumed: the persistence of chloroplast structures that still contain chlorophyllic derivatives slow the chloroplast−chromoplast transition (*2*, *23*). In fact it has been shown that the ultrastructure of the plastids in the ripe stay-green tomato *gf* maintained significant amounts of the chloroplast thylakoid grana along with structures characteristic of the tomato chromoplast. This phenomenon coexists at a molecular level with the retention of plastid photosynthetic proteins (*2*).

Figure 3 shows the electron microscope photographs of plastids from fruit of both genotypes at the ripe stage. Plastids corresponding to the wild genotype (red phenotype) show no thylakoid and high plastoglobuli density (**Figure 3A**) as expected for a fully developed chromoplast, whereas plastids from stay-green fruits have plastoglobuli and thylakoid membranes at the same time (**Figure 3B**), being called chlorochromoplasts. Plastoglobuli are suborganuli specialized in accumulating a high amount of lipids and lipophillic substances such as carotenoids. The chlorochromoplast occurrence has already been described in other *C. annuum* mutants showing overlapped chlorophyll retention during carotenogenesis (*24*). It has been long assumed that during ripening of red fruits of *C. annuum* the chromoplasts are formed in two ways, that is transformation of pre-existing chloroplast and new formation from protoplastids. In the case of stay-green lines the normal chloroplast−chromoplast transition must be altered, as in the case of the *gf* tomato mutant, and the apparently slowed down carotenogenic process must be the result of the physiological adaptation of the fruit to produce mainly new-formed chromoplast in a higher amount than in the red lines, but without modifications in the carotenoid biosynthetic pathway.

Currently, it is generally accepted that there are two main mechanisms involved in the regulation of the biosynthesis and accumulation of chromoplastic carotenoids (*25*). First, the accumulation and increase in carotenoid concentration suggest an increase in the transcripts produced by expression of the carotenoid biosynthesis genes, but also the carotenoid accumulation must be mediated by the presence of carotenoid-storing structures within the plastids. In the present study, plastids for red and stay-green lines show great development of plastoglobuli able to bear carotenoids during carotenogenesis, so that the ability to produce these pigments is related to the expression of the corresponding biosynthetic genes, independently of the retention or catabolism of chlorophylls. In the case of the tomato *gf* mutant, the carotenoid profile resulting from the carotenogenic process related with ripening does not allow distinction between the red and *gf* mutants (*14*).

Chloroplast Environment. Under normal conditions, chlorophylls absorb light and donate electrons to the photosynthetic chain. However, in the absence of enough electron acceptors within the photosynthetic machinery, electrons may be transferred to other compounds, including molecular oxygen, contributing to the formation of harmful free radicals. Therefore, for an adequate photodynamism control, it is essential to keep a correct stoichiometry between chlorophylls and carotenoids

Table 3. Main Carotenoid Indexes of Chloroplast State during the Ripening of Wild-type and Stay-Green Lines of *Capsicum annuum* (L.) Fruits

	ripening stages (days)				
lines	15	30	45	60	75
		Lutein (mg/kg dw)			
Mr 1	96.83	24.96	12.63	0.00	0.00
Mn 3	110.83	33.05	14.92	0.00	0.00
Rr 1	72.78	54.99	22.59	0.00	0.00
Rn 1	58.75	34.97	32.00	0.00	0.00
Rn 2	75.96	61.38	49.06	0.00	0.00
Dr 6	30.61	61.16	0.00	0.00	0.00
Dn 3	71.13	65.48	44.95	0.00	0.00
		β-Carotene (%)			
Mr 1	12.25	6.46	7.59	4.13	5.66
Mn 3	16.54	6.75	8.63	9.38	7.13
Rr 1	17.90	16.45	5.67	6.99	5.20
Rn 1	28.21	14.60	7.58	5.45	4.33
Rn 2	20.49	14.87	6.82	5.33	4.68
Dr 6	17.80	13.39	6.91	5.14	4.50
Dn 3	16.63	15.62	7.98	8.70	4.63
		β-Carotene/Capsanthin Ratio			
Mr 1	-[a]	0.11	0.12	0.06	0.09
Mn 3	-	0.16	0.16	0.20	0.13
Rr 1	-	-	0.10	0.10	0.08
Rn 1	-	-	0.14	0.10	0.08
Rn 2	-	-	0.13	0.10	0.08
Dr 6	-	7.49	0.13	0.09	0.07
Dn 3	-	-	0.16	0.16	0.08

[a] The ratio is impossible because there is not yet capsanthin biosynthesis, only β-carotene. CV $\leq$ 10% in all cases.

within the chloroplastic pigment−protein complexes. In this way, it has been found (*29*) that in the stay-green mutant of *Festuca pratensis* (Huds.) the persistence of chlorophyll is accompanied by both higher photorespiration rates and carotenoid levels, quenching the excess of energy harvested by chlorophylls. Similarly, in the stay-green tomato mutant (*gf*) a smooth rise in the β-carotene/lycopene ratio and percentage of β-carotene has been observed when compared with wild-type tomatoes (*30*). This increase could be related to a physiological mechanism for antioxidant protection of the photosynthetic constituents (*2*). Regarding the studied *Capsicum* cultivars, it has been observed that the relative β-carotene content in respect to the total carotenoid concentration and β-carotene/capsanthin ratio (**Table 3**) tends to be slightly higher in the stay-green lines, as previously observed for Negral cultivar, another chlorophyll-retaining variety (*31*), and the aforementioned *gf* (*30*). The differences are less obvious for ripe fruits of R variety, probably due to less carotenogenesis found in Rr1 line, as mentioned before. Hence, changes in the relative biosynthesis of some individual carotenoid pigments during fruit ripening may contribute to protect the preserved thylakoid structures for longer time in the stay-green lines (**Figure 4**).

Among the chloroplastic carotenoids, lutein stands out as the major one followed by β-carotene and other xanthophylls such as violaxanthin and neoxanthin. In previous studies (*21*, *22*) it has been demonstrated that during ripening of *C. annuum* fruits the typical chloroplast pigments, mainly lutein, gradually disappear and are replaced by typical chromoplast pigments as a result of the down-regulation of genes encoding for ϵ-lycopene cyclase, the enzyme responsible for the formation of the ϵ-ring at one end of lutein. As a result of this process, all the carotenoids present in the ripe fruits belong to the β,β-carotenoid series, the synthesis of which is activated during ripening. In other fruits such as tomato (*Lycopersicon sculetum* Mill.) the activities of β- and ϵ-lycopene cyclase decrease and eventually disappear, resulting in the accumulation of lycopene as the major carotenoid (*32*). Consequently, the analysis of the contents of lutein throughout the ripening process may clarify the relation between the *de novo* carotenogenic process and the retention/catabolism of chlorophylls in stay-green lines. In the present study, and with independence of the phenotype, the lutein content decreases with the progress of the ripening process (**Table 3**) so that the carotenogenic process is similar in the red and stay-green cultivar, suggesting that this process is independent of the retention of chlorophylls in the stay-green ones. We may conclude that the stay-green phenotype is not due to new formed thylakoid structures, since this should be accompanied by lutein biosynthesis as part of the photosynthetic apparatus. In our opinion the stay-green phenotype may be due to some alteration in the PaO activity resulting in a longer persistence of chlorophyll−protein complexes in the thylakoid.

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