Changes in Pigments, Chlorophyllase Activity, and Chloroplast Ultrastructure in Ripening Pepper for Paprika

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Two typical cultivars of pepper for paprika (Bola Roja of red fruits and Negral of brownish fruits) were analyzed for pigments, chlorophyllase activity, and plastid ultrastructure. Three ripening stages were considered: green, unripe (changing color), and fully ripe. Fully ripe peppers of the Negral cultivar retained chlorophyll, while in the Bola Roja fruits this disappeared at the end of maturation. Chlorophyllase activity was higher in the cv. Negral at the first two ripening stages, and similar in both cultivars at the fully ripe stage. Plastids of the early ripening stage were characterized by the typical grana–intergranal structure and a highly developed peripheral reticulum. In the last stage, plastids presented massive structural reorganization. All of these differences among both cultivars are discussed.

Keywords: *Capsicum annuum; carotenoids; chlorophyllase; ultrastructure*

INTRODUCTION

In the study of color changes in fruits, pepper (*Capsicum annuum*) is a very interesting plant material due to the quantity and variety of pigments that are synthesized and the drastic change in color undergone during ripening. At the same time, these fruits undergo wide-ranging modifications of their ultrastructure and chloroplast composition, which is comparable, to a certain extent, to the changes suffered by leaves during senescence, notwithstanding the functional, morphological, and biochemical differences between fruits and leaves (Goldschmidt, 1986). In pepper maturation these ultrastructural changes are accompanied by the transformation of chloroplasts to carotenoid-rich chromoplasts.

According to the bibliography, there are several enzymes that could take part in chlorophyll breakdown. One of the more investigated specific enzymes has been chlorophyllase, which catalyzes the hydrolysis of the phytol chain in chlorophylls and pheophytins to produce chlorophyllides and pheophorbides, respectively (Amir-Shapira et al., 1987; Thomas et al., 1989; Fernández-López et al., 1992). This enzymatic activity has been related to the physiological states of senescence and maturity in leaves and fruits, in an attempt to confirm the decrease of the chlorophyll content with increased activity (Tanaka et al., 1982; Rodríguez et al., 1987; Canjura et al., 1991; Minguez-Mosquera et al., 1993).

With regard to carotenoids, it is well-known that they are situated in the chloroplasts, in the light-harvesting complex, and in the envelope. During fruit ripening there is an accumulation of carotenoids, although not all to the same extent. Luteine, the main xanthophyll in green plants, diminishes and may even disappear, while the content of epoxyxanthophylls increases progressively. These changes are related to the transformation of chloroplasts in chromoplasts which retain the enzymes involved in the carotenogenesis (Gross, 1991). In ripening pepper fruits there is an important accumulation of ketocarotenoids, some of them very specific such as capsanthin, capsorubin, or cryptocapsin. During postharvesting and preservation of the elaborated products (paprika and oleoresin), the carotenoids are subjected to oxidative reactions which involve several enzymes that could also operate on chlorophylls (Barimalaa and Gordon, 1988; Stone and Kinsella, 1989).

In pepper fruits, chromoplasts are organelles developed from protoplast differentiation or, more frequently, by chloroplast transformation (Sitte et al., 1980). It is accepted that the conversion of chloro- to chromoplast is not entirely a degenerative process but the result of a capacity of the plant tissues to achieve a specific metabolic activity (Ziegler et al., 1983). However, how this conversion is controlled is not well understood. The first studies on the ultrastructure of pepper chromoplasts suggested that disintegration of the granal–intergranal network of chloroplasts was the source of the reticular system of the filaments (Frey-Wyssling and Kreutler, 1958) and that this disintegration led to the final lysis of the grana with a parallel increase in the number of plastoglobuli (Spurr and Harris, 1968).

In this paper we study the evolution of the content in photosynthetic pigments and chlorophyllase activity in two cultivars of ripening pepper fruits in an attempt to correlate it with the changes observed in their chloroplast ultrastructure.

EXPERIMENTAL PROCEDURES

Plant Material. Two pepper cultivars, used for paprika production, with different color characteristics were chosen for this investigation: Bola Roja and Negral. Bola Roja is the most typical cultivar in the region of Murcia and is characterized by its spherical form, sweet taste, and bright red color. The Negral cultivar is also spherical and has a sweet taste, but the mature fruits show a brownish tint that diminishes the final quality of the paprika elaborated with these fruits. However, the carotenoid content in the latter cultivar is higher and is excellent for producing oleoresin. Both cultivars are

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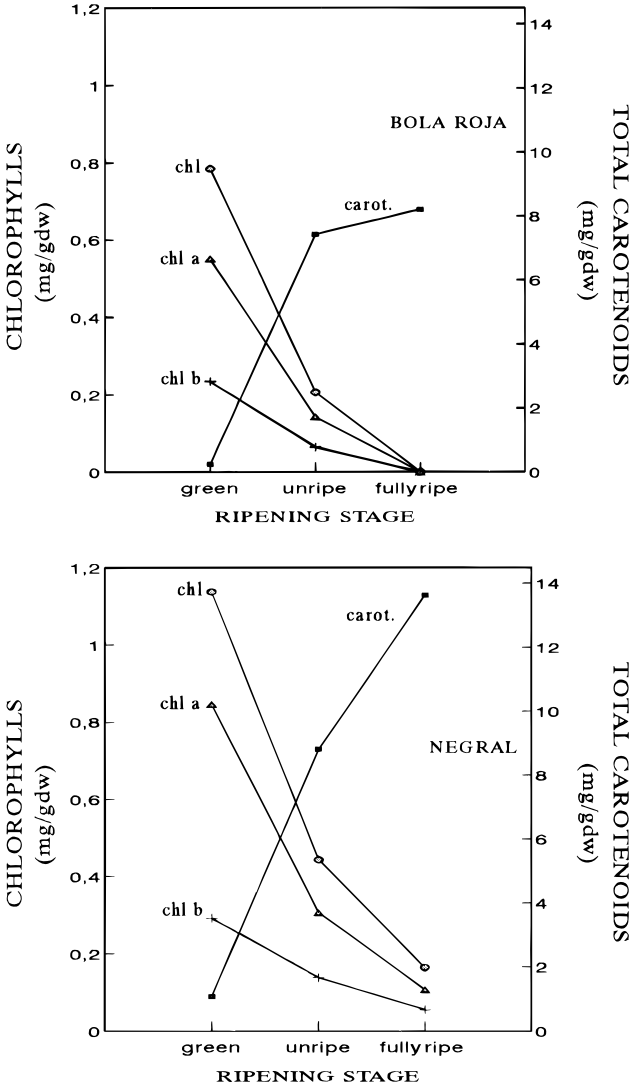

Figure 1. Changes in chlorophylls and total carotenoids during pepper ripening in the cultivars considered (chl *a*, chlorophyll *a*; chl *b*, chlorophyll *b*; chl, total chlorophyll; carot, total carotenoids).

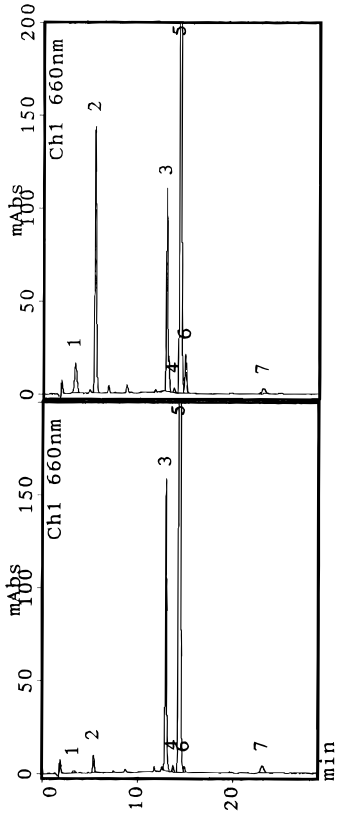

Figure 2. Chromatograms of pepper extracts before (bottom) and after incubation (top). Peak identification: 1, chlorophyllide *b*; 2, chlorophyllide *a*; 3, chlorophyll *b*; 4, chlorophyll *b* epimer; 5, chlorophyll *a*; 6, chlorophyll *a* epimer; 7, pheophytin *a*.

perfectly adapted to the climatic and soil conditions of this area in the southeast of Spain, showing good resistance to rotting. Fruits were harvested at three ripening stages: green, unripe (changing color), and fully ripe. The seeds and peduncles were removed from a 3 kg sample and the peppers were chopped into small pieces and divided in 25 g subsamples which were freeze-dried and stored in vacuo under darkness at −24 °C. In these conditions there was no appreciable alteration in the analytical parameters considered.

Chlorophyll and Carotenoid Content. The chlorophyll content was evaluated by HPLC according to the method proposed previously (Almela et al., 1992). This method permits chlorophyll quantitation in the presence of a certain numbers of carotenoids, as in the case of leaf and green-fruit extracts. However, when the level of carotenoids is higher than the level of chlorophylls and they are esterified with lipids, it is necessary to modify the method by including a previous saponification stage. So, carotenoid extraction, saponification, and HPLC analysis were carried out according to the methods of Almela et al. (1990). A modular Shimadzu (Kyoto, Japan) liquid chromatography system equipped with two LC-6A pumps operated from a SCL-6A controller was used. A SPD-M6A photodiode UV−vis detector operated from the software Class-MXA and a Rheodyne (Cotati, CA) injector (Model 7125) were also employed.

Chlorophyllase Assay. To measure the chlorophyllase activity, we propose an alternative methodology to the classic assay which used acetone powders and the spectrophotometric analysis of the chlorophyllides formed after phase separation (Fernández-López et al., 1992). This method was rather laborious and was susceptible to important errors when the hydrolytic activity was low. We propose a modification of the method of Amir-Shapira et al. (1986). The plant material (25 g) was homogenized in a blender with 50 mL of 0.4 M sucrose in 0.05 Tris-HCl at pH 8.0. The homogenate was filtered through four layers of gauze, and the residue was again homogenized. The filtrates were centrifuged for 10 min at 7000*g* at 4 °C. The pellet, which contained most of the chloroplast material, was suspended in 10 mL of 0.1 M phosphate buffer (pH 8.0) containing 0.2% Triton X-100. The reaction mixture contained 35% acetone, 0.3 mM chlorophylls (Fernández-López et al., 1992), and the chloroplast extract. After incubation in a shaking bath at 30 °C, a 0.5 mL aliquot was transferred to a test tube with 1.0 mL of acetone, where is formed a suspension due to the precipitation of proteins. This suspension was sonicated and centrifuged at 12000*g* for 5 min. The pigment extract obtained was passed through a 0.2 μm filter and analyzed by HPLC. To optimize the HPLC separation of free phytol chloropigments, an ODS column (25 cm × 0.4 cm i.d.) of 5 μm spherical particles (Spherisorb ODS-2) was used. The pigments were eluted using a linear gradient from 80% methanol in 1 M ammonium acetate to 80% methanol in acetone for 10 min. This final composition was maintained until the pigments were completely eluted. The flow rate was 1 mL/min.

Protein Content. This was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Electron Microscopy. The plant tissue was fixed in 2% glutaraldehyde and postfixed in 1% osmium tetraoxide. The fixed material was embedded in Spurr resin, and thin sections were stained with uranyl acetate and lead citrate and then examined under a Zeiss EM-9 electron microscope.

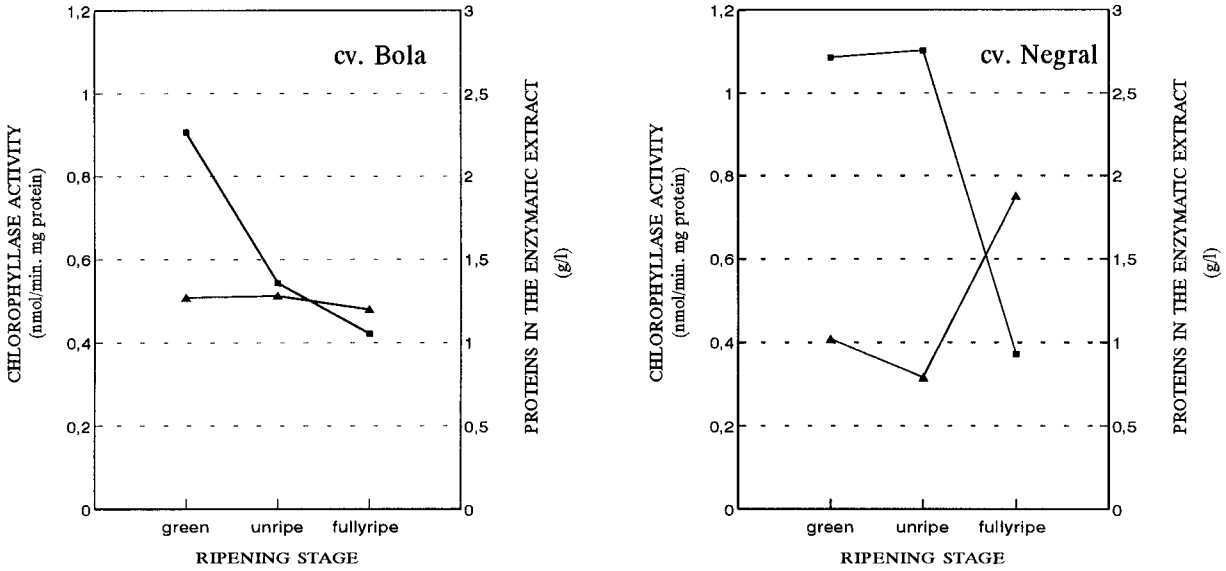

Figure 3. Changes in chlorophyllase activity in the cv. Negral and in the cv. Bola Roja. Squares represent the enzymatic activity evaluated as total chlorophyllide; triangles represent the protein content in the enzymatic extract.

RESULTS AND DISCUSSION

Ripening-associated changes in the chlorophyll content of the pepper fruits are shown in Figure 1, in which the total carotenoid content is also included to indicate the degree of maturity. It can be observed that the Negral variety showed a higher chlorophyll content in the green and unripe stages, although the most significant observation was the important quantity of chlorophyll retained in the fully ripe stage, almost 14% of the chlorophylls noted in the green stage. However, in the Bola Roja fruits, the chlorophylls disappeared in the final ripening stage. This different evolution in the chlorophyll content is closely related with the chloroplast ultrastructure in both cultivars.

Chlorophyllase activity was evaluated by measuring the chlorophyllides formed after incubation of the chloroplast fragments, so as to avoid possible errors that could be introduced if the chlorophyll substrate were measured, the diminution of which might be the result of a mechanism other than this enzyme activity. Figure 2 shows the chromatograms corresponding to the initial time and after incubation in the previously described conditions. The good resolution between peaks can be appreciated. No experiment formed pheophytins or other magnesium-free catabolites. A slight increase in the content of chlorophyll epimers could be observed as consequence of incubation to 30 °C.

The HPLC method used to evaluate the products of the enzymatic reaction is rapid, precise, and reproducible, avoiding two errors inherent to the spectrophotometric methods: (a) considering the epimerization like enzymatic alteration and (b) the phase separation, which is necessary when spectrophotometric methods are employed.

The facts that detergents are necessary for enzyme solubilization and there is an increased activity obtained when acetone is employed offer support to the hypothesis that the enzyme and its substrate are structurally separated within the membrane in a way which does not permit the hydrolysis of chlorophyll to occur. Disruption of the thylakoid membrane by mechanical means or by adding detergents would then bring the enzyme and substrate into contact, thus initiating chlorophyll degradation (Lambers et al., 1984; Rodriguez et al., 1987).

Figure 3 shows the chlorophyllase activity in both varieties studied. Bola Roja fruits showed a lower activity as maturation increased. Negral fruits showed a similar activity in the first two stages [ca. 1.1 nmol of chlorophyllide min^{-1} (mg of protein)$^{-1}$], possibly due to the lower degree of disorganization of the chloroplast structure in this variety at the beginning of maturation, as could be observed in the ultrastructural study.

The hydrolytic activity of the enzyme with chlorophyll *a* was between 2.7 and 3.1 times higher than with chlorophyll *b*. This result should not be interpreted to mean that chlorophyll *a* is a preferred substrate for chlorophyllase because the concentrations of chlorophylls *a* and *b* were different in the reaction mixture (chl *a*/chl *b* ratio of approximately 2.5). Similar results were obtained in olives (Minguez-Mosquera et al., 1993); however, in satsuma fruits a preferred hydrolysis of chlorophyll *b* has been reported (Shimokawa, 1979).

Table 1 presents the carotenoid composition of both cultivars during maturation, with the sharp qualitative and quantitative changes that take place. Lutein, the principal xanthophyll of leaves and green fruits, disappears. Carotenes, which are situated at the beginning of the final stages of the biosynthetic pathway and which are plentiful in the green stage, diminish drastically when the fruits are fully ripe and are transformed into more oxygenated xanthophylls at the end of the pathway, mainly capsanthin.

Carotenoid evolution is not the same in both varieties. Cv. Bola Roja accumulates a higher percentage of capsanthin and capsorubin than cv. Negral. Negral fruit retains a certain level of lutein even in the fully ripe stage, which would imply a minor evolution of the plastids in this variety that is revealed by the presence of chlorophyll in mature fruits, higher chlorophyllase activity, and a carotenoid makeup which is less evolved that in classical red varieties.

It is clear that in pepper fruits an important number of epoxides occur naturally, and it has been postulated that the formation of epoxides is the initial step in carotenoid breakdown (Simpson and Chichester, 1981). Camara and Moneger (1982) proposed a sequence for the interconversion of xanthophylls in *Capsicum*, although cryptocapsin is not included in their scheme; however, given its structural similarity with capsanthin,

Table 1. Individual Carotenoid Content (Percent) in the Saponified Extracts of the Pepper Cultivars Analyzed[a]

	cv. Bola Roja			cv. Negral		
pigment	green	unripe	fully ripe	green	unripe	fully ripe
β-carotene	23.12 ± 0.63	9.39 ± 0.30	6.06 ± 0.21	23.88 ± 0.72	11.84 ± 0.39	7.53 ± 0.21
cryptocapsin	35.56 ± 1.17	8.04 ± 0.27	0.53 ± 0.03	27.55 ± 0.78	9.62 ± 0.27	0.71 ± 0.03
cryptoflavin	2.11 ± 0.09	3.72 ± 0.09	4.76 ± 0.15	2.93 ± 0.24	4.02 ± 0.12	5.94 ± 0.18
β-cryptoxanthin	5.14 ± 0.15	2.83 ± 0.12	2.01 ± 0.09	5.02 ± 0.15	3.24 ± 0.09	2.31 ± 0.09
antheraxanthin	1.23 ± 0.06	0.74 ± 0.03	0.92 ± 0.06	1.46 ± 0.06	1.34 ± 0.03	1.83 ± 0.06
lutein	2.34 ± 0.09	0.36 ± 0.03		2.81 ± 0.12	1.45 ± 0.06	1.36 ± 0.06
capsolutein	4.03 ± 0.27	3.88 ± 0.12	6.08 ± 0.21	4.82 ± 0.15	4.31 ± 0.15	5.97 ± 0.18
luteoxanthin	1.74 ± 0.06	1.92 ± 0.09	2.53 ± 0.09	1.53 ± 0.06	1.86 ± 0.06	2.96 ± 0.12
zeaxanthin	2.02 ± 0.09	4.68 ± 0.15	4.11 ± 0.12	1.96 ± 0.09	1.84 ± 0.06	3.25 ± 0.09
mutatoxanthin	1.84 ± 0.06	3.04 ± 0.12	2.75 ± 0.09	0.62 ± 0.03	3.71 ± 0.12	5.06 ± 0.18
capsanthin	6.92 ± 0.24	35.08 ± 1.11	46.91 ± 1.50	8.36 ± 0.24	32.52 ± 1.05	38.16 ± 1.17
capsathin 5,6-epoxide	0.33 ± 0.03	2.76 ± 0.09	3.64 ± 0.12	1.66 ± 0.06	1.28 ± 0.06	2.56 ± 0.09
violaxanthin	1.01 ± 0.06	0.93 ± 0.06	0.61 ± 0.03	1.04 ± 0.06	4.51 ± 0.15	3.92 ± 0.12
capsorubin	0.28 ± 0.03	5.84 ± 0.15	6.52 ± 0.21	0.94 ± 0.06	5.97 ± 0.12	5.87 ± 0.15
capsorubin isomer	0.23 ± 0.03	5.62 ± 0.18	6.08 ± 0.21	0.51 ± 0.03	4.81 ± 0.15	4.77 ± 0.18
neoxanthin	0.56 ± 0.03	3.11 ± 0.09	3.41 ± 0.12	1.86 ± 0.06	4.16 ± 0.12	4.32 ± 0.12
unknown	6.00 ± 0.45	8.06 ± 0.54	3.08 ± 0.24	7.05 ± 0.45	4.45 ± 0.36	2.59 ± 0.05

[a] Data are means of three analyses ± SD.

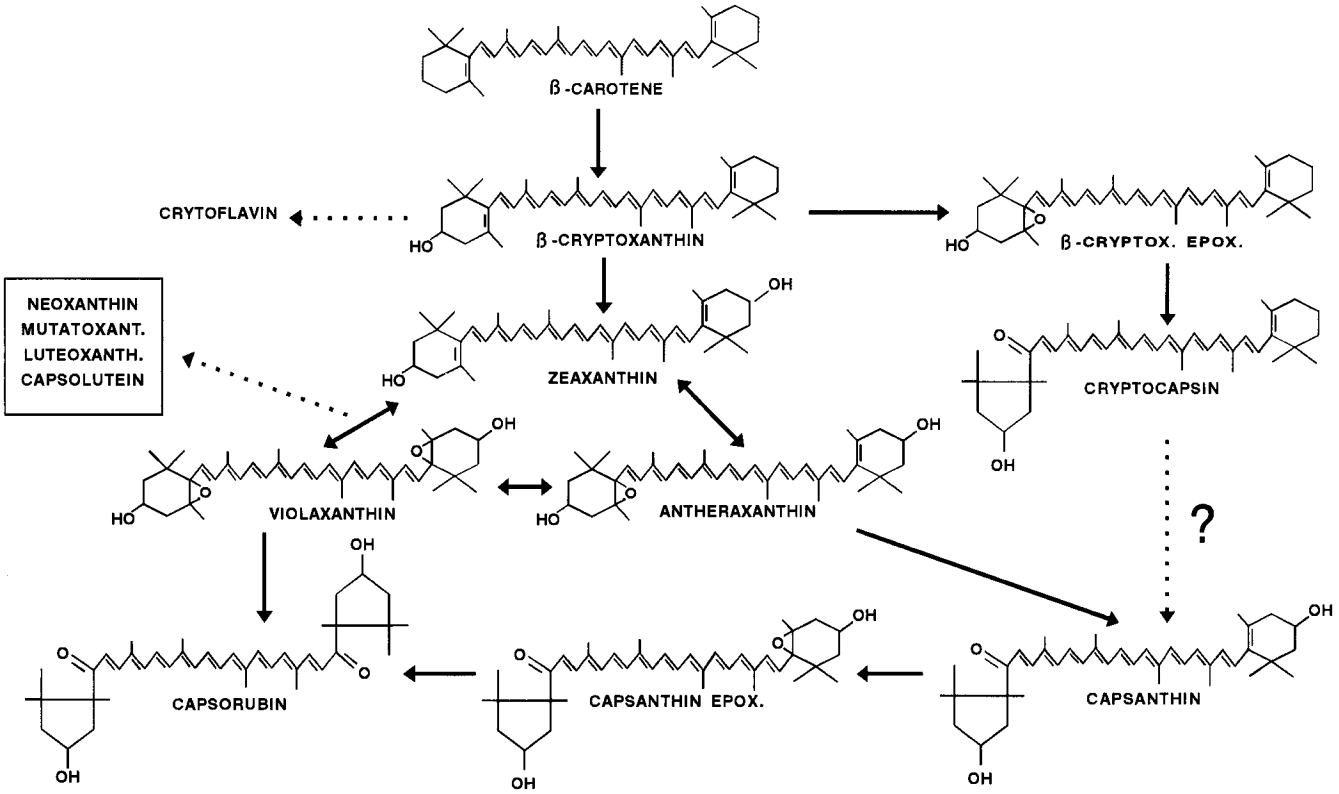

Figure 4. Scheme for some steps of carotenoid synthesis.

a relationship with its pathway cannot be discounted. Figure 4 summarizes a scheme that might explain the last steps of carotenoid synthesis.

It is accepted that carotene hydroxylation occurs after external ring closure (Britton, 1976), so β-cryptoxanthin and zeaxanthin arise from β-carotene after hydroxylation in C-3 and C-3,3′ positions, respectively, with retention of configuration. For epoxide formation the initial site of attack is the 5,6-double bond (El-Tinay and Chichester, 1970). The conversion of zeaxanthin into antheraxanthin and violaxanthin involves the formation of 5,6-epoxides. Neoxanthin can arise from zeaxanthin after the formation of 5,6-epoxides, which are later converted into an allenic end group (Britton, 1982). Other pigments such as mutatoxanthin and luteoxanthin, with 5,6- and 5,6−5′,8′-epoxide groups, could derive from the pool formed between zeaxanthin and anteraxanthin.

There is strong evidence to support the hypothesis that antheraxanthin and violaxanthin are involved in the formation of the ketoxanthophylls capsanthin and capsorubin. The mechanisms suggested involve a pinacolic rearrangement (Camara, 1980; Camara and Moneger, 1981; Rüttiman, 1982; Afitlhile et al., 1993). Cryptocapsin, capsanthin, and capsorubin belong to the red pigment group of *Capsicum* fruits and are rarely found in other sources. Their structural similarity and their presence as the characteristic pigments in the fruits of the *Capsicum* class suggest that there may be a biosynthetic relation between them.

Tables 2 and 3 show the correlation between the carotenoids situated at the beginning and at the end of the biosynthetic pathway. The values described were obtained from the Pearson matrix calculated from the pigment concentrations observed during maturation.

Table 2. Correlations among Carotenoid Pigments of the Cv. Negral

	β-carot	β-cryptox	cryptocap	capsant	capsorub	neoxanth	mutatox	luteoxanth
β-carot								
β-cryptox	0.996							
cryptocap	0.997	0.999						
capsant	−0.997	−0.986	−0.988					
capsorub	−0.963	−0.935	−0.940	0.981				
neoxanth	−0.980	−0.959	−0.963	0.993	0.997			
mutatox	−0.999	−0.999	−0.999	0.993	0.950	0.971		
luteoxanth	−0.843	−0.887	−0.881	0.799	0.666	0.720	0.866	
cryptofla	−0.911	−0.944	−0.940	0.876	0.766	0.812	0.929	0.990

Table 3. Correlation among Carotenoid Pigments in the Cv. Bola Roja

	β-carot	β-cryptox	cryptocap	capsant	capsorub	neoxanth	mutatox	luteoxanth
β-carot								
β-cryptox	0.998							
cryptocap	0.999	0.999						
capsant	−0.994	−0.999	−0.996					
capsorub	−0.996	−0.988	−0.994	0.982				
neoxanth	−0.996	−0.987	−0.994	0.981	0.999			
mutatox	−0.914	−0.883	−0.905	0.865	0.945	0.946		
luteoxanth	−0.800	−0.840	−0.812	0.860	0.746	0.744	0.487	
cryptofla	−0.977	−0.990	−0.981	0.994	0.955	0.954	0.806	0.910

A high linear correlation between β-carotene, β-cryptoxanthin, and cryptocapsin can be observed in both varieties, which endorses the hypothesis that they evolve in the same way, being intermediaries of carotenogenesis in the varieties studied. We also obtained high correlations, but negative, between these three carotenoids and the xanthophylls situated at the end of the pathway (capsanthin, capsorubin, neoxanthin, mutatoxanthin, luteoxanthin, and cryptoflavin). This suggests that the synthesis of these compounds during maturation is paralleled by a decrease in β-carotene, β-cryptoxanthin, and cryptocapsin, which are possible precursors in *Capsicum*.

The three xanthophylls included in the violaxanthin cycle (antheraxanthin, zeaxanthin, and violaxanthin) (Siefermann-Harms, 1977) showed no significant correlation with each other.

Figure 5 presents the relationship between the main carotenoid of pepper (capsanthin) and its possible precursors: antheraxanthin, zeaxanthin, and violaxanthin. It can be appreciated that the relative evolution of capsanthin with regard to the pigments of the violaxanthin cycle is not representative. However, if we consider also the level of cryptocapsin, there is a close relation between the decrease of these four carotenoids and the increase in capsanthin. These results agree with those reported in other pepper varieties (Almela et al., 1991).

Very little is known about the biochemical changes that take place during chloroplast−chromoplast transformation in contrast to the rapidly increasing amount of knowledge concerning processes during the development of etioplast into chloroplast. In the last part of this paper, therefore, we describe the evolution of the plastid ultrastructure in Bola Roja and Negral peppers during maturation and attempt to correlate it with the presence of photosynthetic pigments.

Figure 6 shows the electron micrographs of both varieties in the green stage. Note the well-developed plastids with a clearly defined thylakoid network and many grana stacks. The stroma contains several starch grains. The most significant difference with respect to the green tissues of higher plants is the greater thylakoid size and lower degree of aggregation. Camara and Brangeon (1981) reported very similar observations in Yolo Wonder peppers.

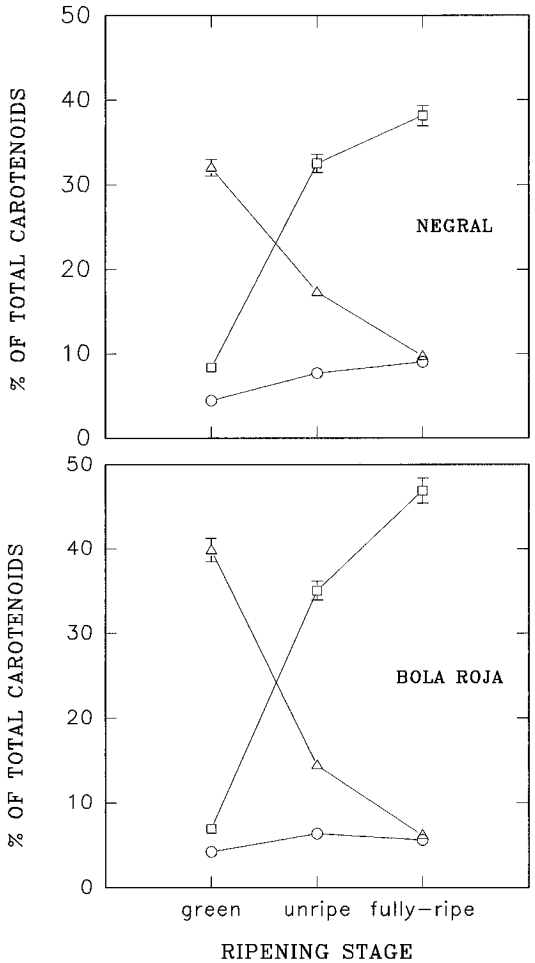

Figure 5. Changes in capsanthin content (squares), violaxanthin plus antheraxanthin plus zeaxanthin content (circles), and violaxanthin plus antheraxanthin plus zeaxanthin plus cryptocapsin content (triangles), in Negral and Bola Roja peppers. The standard deviations of the means are shown, except when they are smaller than the symbol.

The plastids in the transition stage (unripe) are shown in Figure 7. In Bola Roja fruits grana disappear and starch grains and extended thylakoids can be observed. According to the bibliography, the thylakoids derive from the inner envelope membrane rather than from the lysis or disintegration of grana (Camara and

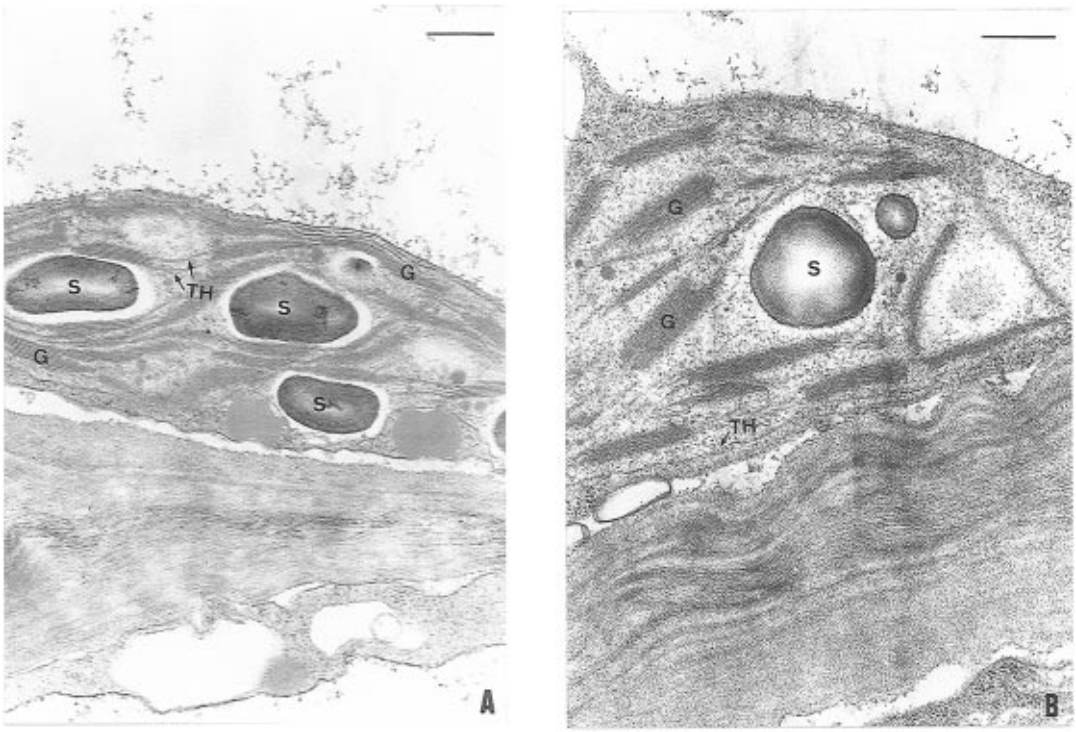

Figure 6. Electron micrographs corresponding to plastids from the green ripening stage in pepper fruit cv. Bola Roja (A) and cv. Negral (B). S, starch; TH, thylakoid; G, grana. Bars represent 0.5 μm.

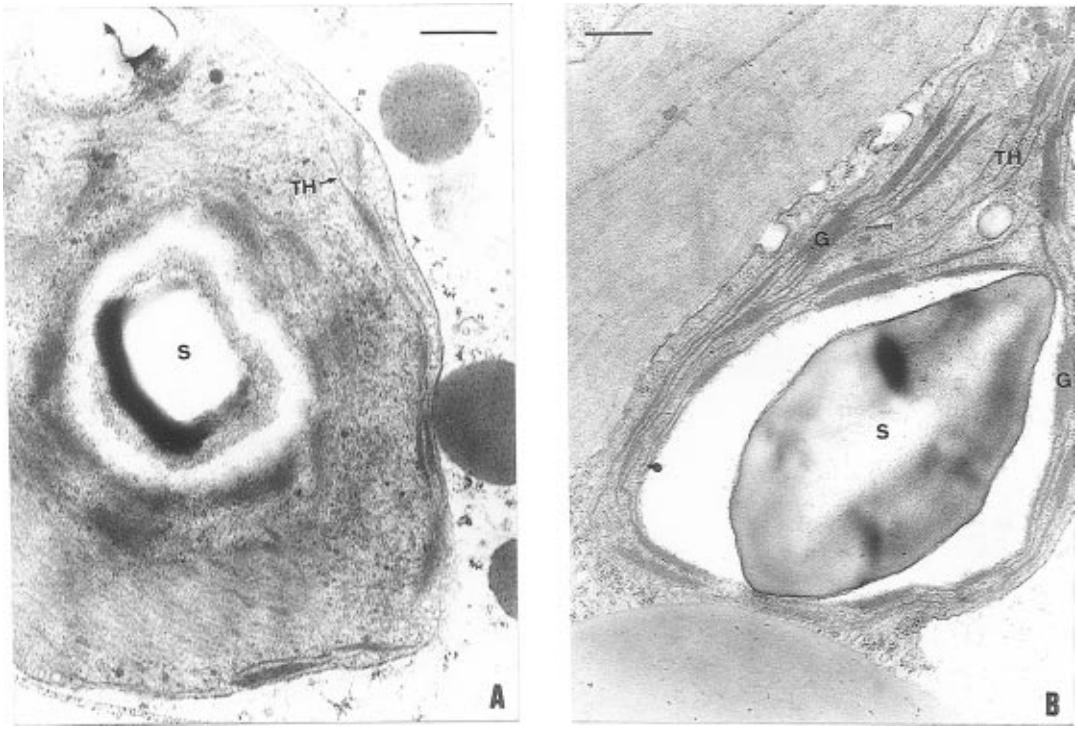

Figure 7. Electron micrographs corresponding to plastids from the unripe ripening stage in pepper fruit cv. Bola Roja (A) and cv. Negral (B). S, starch; TH, thylakoid; G, grana. Bars represent 0.5 μm.

Brangeon, 1981; Camara, 1985; Carde et al., 1988). Chloroplast disorganization in Negral fruits is less and well-structured grana are present.

In the fully ripe stage (Figure 8), Bola Roja fruits present massive structural reorganization. The formless plastids show peripheral tubular structures and microtubullar structures in the form of reticulum (Lawrence and Possingham, 1984; Carde et al., 1988). Plastids of the Negral variety show the characteristics of a well-organized chromoplast; grana have disappeared, but they show an important peripheral tubular network that in some cases seems slightly stacked. A large number of plastoglobuli can be observed.

Plastid evolution during the ripening of *C. annuum* fruits is consistent with their gradual loss of chlorophyll. Hence, the persistence of long peripheral thylakoids in the chromoplasts of Negral peppers is probably associated with the presence of chlorophyll in fully ripe fruits of this variety. Experiments carried out with leaves, in which senescence or nutritional stress is induced, show similar characteristics with a low starch content, a lower degree of thylakoid stacking, and a higher

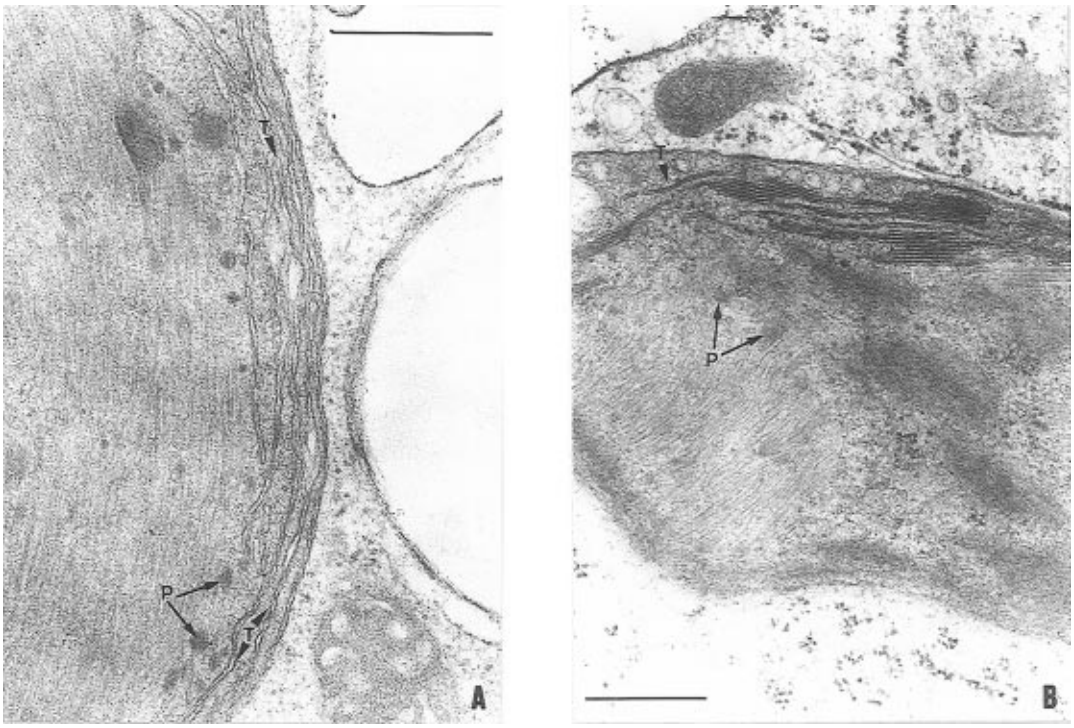

Figure 8. Electron micrographs corresponding to plastids from the fully ripe ripening stage in pepper fruit cv. Bola Roja (A) and cv. Negral (B). P, plastoglobuli; T, tubules. Bars represent 0.5 μm.

proportion of unappressed membranes (Pushnik and Miller, 1982; Hech-Buchholz, 1983; Biswall and Biswall, 1988; Takács and Técsi, 1992).

In higher plants carotenoid biosynthesis occurs within the plastids. The first studies supported the localization of the carotenoid biosynthesis enzymes within the inner envelope of plastids (Lutke-Brinkhaus et al., 1982). However, it is now widely accepted that this enzymatic activity is associated with the thylakoids (Dogbo et al., 1987; Serrano et al., 1990; Sandmann, 1991; Linden et al., 1993), but the breakdown of chloroplast structures has not been determined. The fact that plastids maintain their biosynthesic capacity even in the fully ripe stage suggests that the tubular systems observed play an important role. Thus, the qualitative and quantitative differences observed between the two pepper varieties, especially with regards to the final carotene and ketoxanthophyll contents, would be associated with the different developments of the membrane systems.

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