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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Carotenoid-bearing structures in fruit chromoplasts of *Solanum capsicastrum* Link.

NIKOLA LJUBEŠIĆ[1]*, MERCEDES WRISCHER[1], TATJANA PREBEG[1], DRAGOMIR BRKIĆ[2]

[1] Ruđer Bošković Institute, Department of Molecular Genetics, PO Box 180, HR-10002 Zagreb, Croatia

[2] Faculty of Pharmacy and Biochemistry, Department of Pharmaceutical Botany, Schrottova 39, HR-10000 Zagreb, Croatia

The fine structure, pigment content and protein profile of chromoplasts in fruit of *Solanum capsicastrum* were studied during the various stages of ripening. Plastid metamorphosis is synchronized with the disappearance of chlorophylls and intense synthesis of carotenoids. The chloro-chromoplasts observed in the first stages of fruit ripening accumulated large groups of plastoglobules. As the ripening process proceeded, the plastoglobules transformed into small crystalloids and tubules. In red ripe fruit the chromoplasts contained three carotenoid-bearing structures: plastoglobules, small crystalloids and tubules. Fruit treated with norflurazon formed only numerous plastoglobules, whereas the other two structures were absent. The results are discussed with regard to previous results on the formation of specific chromoplasts structures.

Key words: *Solanum capsicastrum*, fruit chromoplast, carotenoid-bearing structure, ultrastructure

Introduction

Chromoplasts of fruits and flowers are characterized by a very heterogeneous ultrastructure. A large variety of carotenoids are stored in specific structures: the plastoglobules, chromoplast internal membranes, tubules, and carotenoid crystalloids (SITTE et al. 1980, LJUBEŠIĆ et al. 1991, CAMARA et al. 1995). Several of these structures may appear during chromoplast differentiation either simultaneously or in succession. In the chromoplasts of ripening fruit of *Solanum capsicastrum* three of these structures were described: plastoglobules, crystalloids and tubules (SIMPSON et al. 1978). Here we report on the relation and succession of these structures during chromoplast differentiation. Ultrastructural data are supplemented by pigment and protein analyses. The effect of norflurazon, a specific

* Corresponding author: Tel. (385 1) 4680 238, e-mail: ljubesic@rudjer.irb.hr

inhibitor of carotenoid synthesis, provides further insight into the mechanism of the observed ultrastructural changes.

Materials and Methods

Plants of *Solanum capsicastrum* Link. were grown under greenhouse conditions. For analysis, fruit in different ripening stages were used. Dark-green, fully developed fruit were used for treatment with norflurazon. The fruit were treated 5 days with $2 \cdot 10^{-4}$ M and $2 \cdot 10^{-5}$ M aqueous solution of norflurazon and used for analysis after 3 and 4 weeks.

For light microscopic investigations hand-made sections of fresh tissue cut with a razor blade were kept in 0.1 M sucrose solution and examined with a Zeiss Axiovert 35 light microscope and a Zeiss Axiolab polarizing microscope.

For electron microscopic investigations pieces of tissue were fixed in 1% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 30 min. After washing in buffer, they were postfixed in 1% OsO_4 for 2 h. The dehydrated tissue was embedded in araldite or in Spurr's medium. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 10A electron microscope (WRISCHER et al. 1999). For visualization of glycolipids the thiocarbohydrazid-Ag-proteinate or thiocarbohydrazide-OsO_4 procedures were applied (WRISCHER et al. 2001).

Pigments were extracted with 100% acetone and quantified spectrophotometrically according to LICHTENTHALER (1987). For qualitative analysis the pigments were separated by thin-layer chromatography on silica-gel G plates in a mixture of petroleum ether (40–70 °C) and acetone (70:30) and identified by their absorption maxima in a number of solvents.

For protein analysis, pericarp tissue was chopped in a solution for protein denaturation (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate, 0.1% bromphenol blue) with addition of polyvinylpyrolidone and quartz sand, and centrifuged at 10 000 g for 15 min. The proteins in the supernatant were denatured for 5 min at 80 °C and separated using 12.5% SDS-PAGE (LAEMMLI 1970) and staining with Coomassie brilliant blue R-250.

Results

Macroscopic observations

The fully grown, dark-green, unripe fruit had about 1 cm in diameter. During the ripening process, which required several weeks, colors changed from yellow-green, through yellow and orange, to red. The norflurazon-treated fruit started to yellow after 2 weeks, were completely yellow after 3 weeks, and remained without changes in color till their withering.

Light microscopy

The cells of green fruit tissue contained large chloroplasts with starch grains and dark-green grana structures. In yellow-green and yellow fruit the plastids were variable in shape. Some of them were oval and some elongated with inclusions in the stroma that showed birefringence under the polarizing microscope. In the cells of orange and red fruit the chromoplasts were oval or crescent-shaped. Dark-red inclusions were visible in their stroma.

After 3 weeks of norflurazon treatment only spherical pale-yellow chromoplasts were visible. No particular internal structures could be observed. Birefringence and dichroitic areas under the polarizing microscope were absent.

Electron microscopy

The ultrastructure of the plastids in the subepidermal and deeper cell layers of the fruit pericarp was identical. In dark-green fruit the chloroplasts had a normal ultrastructure. Large grana were connected with stroma thylakoids and in the stroma there were starch grains, as well as some small dark plastoglobules.

Striking changes in plastid morphology were already detected in yellow-green fruit. The number of grana was reduced; frequently only single thylakoids remained. Large groups of plastoglobules, 0.1–0.2 μm in diameter, had formed in the stroma, while starch had disappeared (Fig. 1). In some cells grana were present, but their thylakoids had highly dilated lumina (Fig. 2). During further yellowing of the fruit the thylakoid system was reduced to short membrane vesicles. At the same time, plastoglobules became numerous.

In the chromoplasts of orange fruit, lightly stained inclusions appeared in some plastoglobules (Fig. 3). Some of these inclusions, which protruded into the stroma, were straight and rigid. By their regular edges, they were recognized as crystalloids, about 0.2–0.4 μm long and 10–30 nm thick (Fig. 3). At first the crystalloids were in contact with plastoglobules, but later some were also found lying free in the stroma (Fig. 4). At this time, or slightly later, tubules appeared in the stroma (Fig. 4). The tubules were several μm long, roundish, 20–25 nm in cross-section (Fig. 4). They were often aggregated into groups arranged along the long axes of the elongated chromoplasts. Attachment of the tubules to membranes was not detected with certainty.

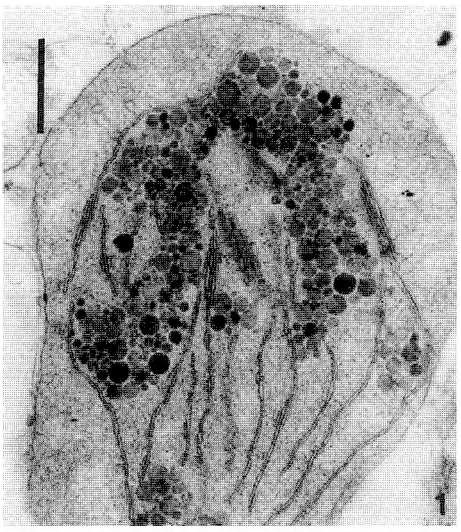
In red fruit, contact of the tubules with plastoglobules was now visible. By the TCH-Ag-proteinate or the TCH-OsO_4 procedure only glycolipids could be stained. Therefore the ultrastructure of the tubules and their contact with the plastoglobules was clearly seen. Some pictures indicated that the tubules may grow from the plastoglobules (Figs. 5, 6). By this staining it became also well visible that some tubules were glued together in groups of two or more, a phenomenon most clearly observed in cross-sections (Fig. 7).

In the chromoplasts of ripe dark-red fruit, the remains of some tubules and crystalloids were irregularly dispersed in the empty stroma, while the plastoglobules were reduced in size and number.

In norflurazon treated yellow fruit, 4 weeks after the start of the treatment, except for some long single thylakoids, there were only accumulation of numerous plastoglobules of low osmiophilic (Fig. 8). Rarely some short tubules or structure like the low osmiophilic globules (WRISCHER et al. 1999) were found in the stroma. These structures were probably formed or were in process of formation before treatment with norflurazon.

Pigment and protein analysis

Dark-green fruit contained high amounts of chlorophylls and of carotenoids. During ripening the content of chlorophyll gradually decreased, while that of carotenoids constantly increased. The process of ripening was followed by thin-layer chromatography. Of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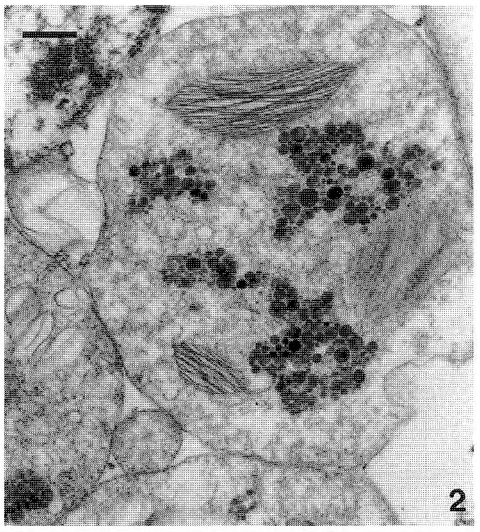
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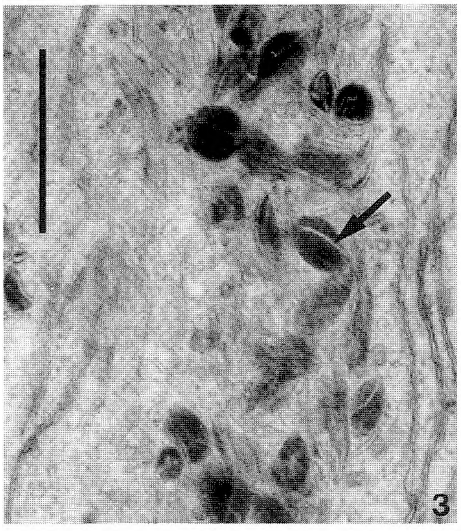
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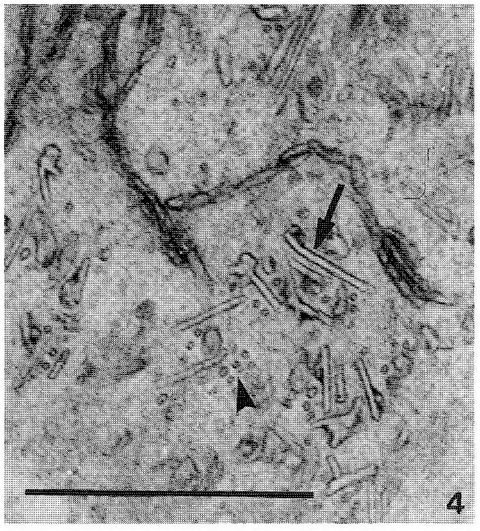
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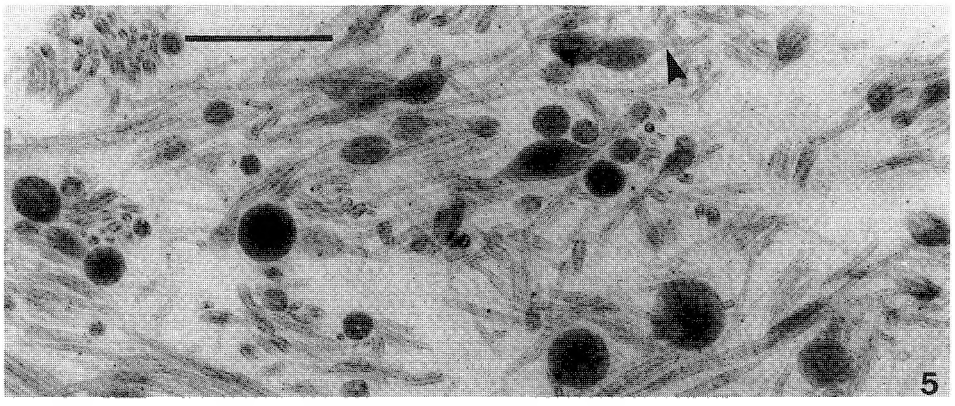
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total carotenoids in the control red fruit 51.3% were ß-carotene, 34% cryptoxanthin, and 14.5% lutein.

Norflurazon-treated fruit contained only carotenoids, without any traces of chlorophylls. Red fluorescence on chromatograms indicated the presence of small amounts of pheophytin. The concentration of carotenoids was 11 times lower (0.174 mg · g^{-1} fr. wt.) than in control red fruit (Tab. 1). The carotenoid ratios (β-carotene to cryptoxanthin and lutein) were similar as in the control red fruit. However, thin-layer chromatography showed the presence of traces of an unknown carotenoid with an approximate R_f value of about 0.8.

Tab. 1. Content of chlorophylls and carotenoids (mg · g^{-1} fresh weight) during the ripening of control and treated fruit.

	Control				Norflurazon
	Green	Yellow-green	Orange	Red	Yellow
Total chlorophylls	0.813	0.213	0.035	–	–
Carotenoids	0.169	0.246	0.277	1.930	0.174

The SDS-PAGE analysis in red control fruit indicated the presence of a polypeptide of about 32 kDa. The molecular weight suggests that this polypeptide is fibrillin (DERUÈRE et al. 1994).

Discussion

In the majority of plants, the chromoplasts contained only one, or one prevailing, characteristic carotenoid-bearing structure (LJUBEŠIĆ et al. 1991). In the ripe fruit of *Solanum capsicastrum* three chromoplasts structures appeared together: plastoglobules, crystalloids and tubules. Although plastoglobules were more numerous in the earlier stages and crystal-

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Figs. 1 – 7. Differentiation of chromoplasts in untreated fruit of *Solanum capsicastrum* Link. Figs. 1 – 4 and 8: standard preparation; Figs. 5 – 7: glycolipid-staining. Bars = 0.5 µm.

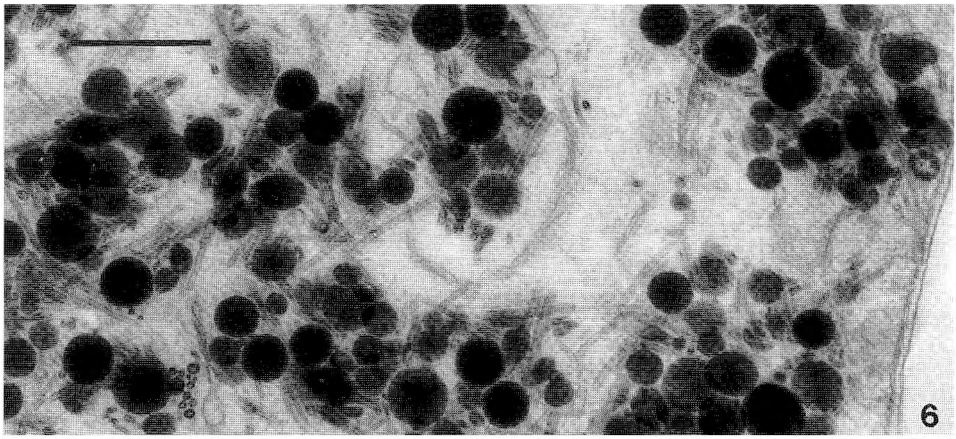
Fig. 1. Chloro-chromoplast from a yellow-green fruit. In the stroma there are aggregates of plastoglobules and mostly single thylakoids.

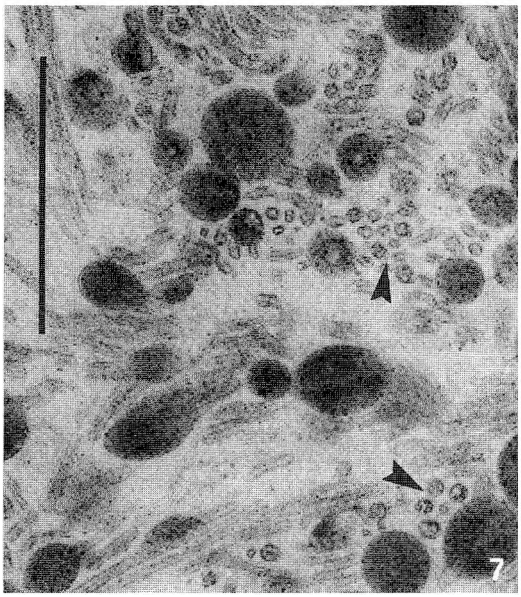
Fig. 2. Chloro-chromoplast from a yellow-green fruit. In addition to groups of plastoglobules there are grana with highly dilated thylakoid lumina.

Fig. 3. Part of a chromoplast from an orange fruit. Some plastoglobules contain crystalloid inclusions (arrow).

Fig. 4. Part of a chromoplast from an orange fruit with crystalloids (arrow) and cross-sectioned tubules (arrowhead).

Fig. 5. Part of a chromoplast from a red fruit. Some tubules are in contact with plastoglobules (arrowhead). TCH-Ag-proteinate-staining.





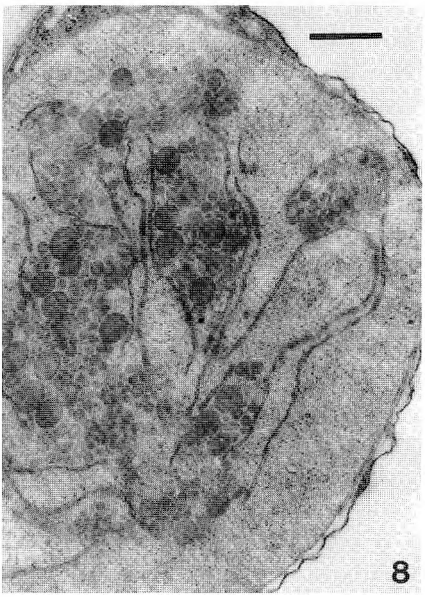


Fig. 6. Numerous plastoglobules and tubules in the chromoplast from a red fruit. TCH-OsO_4-staining.

Fig. 7. The same object as in Fig. 5. Some of the cross-sectioned tubules are stuck together (arrowheads). TCH-Ag-proteinate-staining.

Fig. 8. Plastid from a norflurazon treated yellow fruit with numerous plastoglobules and single thylakoids. Standard preparation. Bar = 0.5 μm.

loids and tubules in the late stages, all three structures existed during whole period of fruit ripening. The origin and differentiation of these three structures in *S. capsicastrum* are closely connected. Chromoplast tubules originate at least in two ways: from plastoglobules and from various membrane structures (LJUBEŠIĆ et al. 1995; WRISCHER et al. 1998, 1999;

PREBEG 2000). Our data suggest that the tubules in *S. capsicastrum* originate from plastoglobules. The crystalloids could be formed from plastoglobules or in the lumen of thylakoids (KUHN 1970, HLOUŠEK-RADOJČIĆ and LJUBEŠIĆ 1988). The small crystalloids in ripe *S. capsicastrum* develop only from plastoglobules. Why of none of these structures prevail in mature chromoplasts is unclear. It is possible that the rate of carotenoid synthesis was not sufficient for the formation of numerous crystalloids.

SDS-PAGE analysis showed the presence in red fruit very small amounts of a polypeptide of about 32 kDa. We believe that this polypeptide represents fibrillin, which is the protein main component of chromoplast tubules (LJUBEŠIĆ et al. 1996, VISHNEVETSKY et al. 1999, WRISCHER et al. 1999). Small level of fibrillin maybe limits large accumulation of fibrils in chromoplasts of *S. capsicastrum.* Biochemical and structural studies by other authors indicate that the type of carotenoid-bearing structure is governed by the quantitative and qualitative ratio of lipids, proteins and carotenoids (DERUÈRE et al. 1994). We suppose that a specific balance of small amounts of fibrillin and intermediate concentrations of certain carotenoids and lipids induce the simultaneous formation all three characteristic chromoplasts structures in chromoplasts of *S. capsicastrum.*

Our observations on the dimensions and morphology of the small crystalloids corroborate prior reports (SIMPSON et al. 1978, LJUBEŠIĆ et al. 1999). The thickness of the crystalloids varied from 10 to 30 nm and their length from 0.2 to 0.4 μm. Sections through the crystalloids indicate that they are straight-sided slabs, which were separated from one another and from the stroma by dark-staining envelopes (membranes?). In earlier developmental stages, the crystalloids are often associated with remains of electron-dense plastoglobuli material.

The composition of the small crystalloids was not analyzed by chemical methods. However, the origin and morphological similarity to analogous structures in the chromoplasts of the fruit of *Physalis alkekengi,* in which β-carotene is predominant carotenoid (84.4%), suggests that the small crystalloids are mostly β-carotene (LJUBEŠIĆ et al. 1999). The percentage of β-carotene in red fruit of *S. capsicastrum* (51.3%) containing small crystalloids, supported the possibility of the β-carotene nature of these structures. The absence of small crystalloids in yellow fruit treated with norflurazon, which is known to be a specific inhibitor of β-carotene biosynthesis, supports this assumption (EMTER et al. 1990, SALOPEK and LJUBEŠIĆ 1994, WRISCHER et al. 1998, LJUBEŠIĆ et al. 1999). The finding of some authors that the small crystalloids in *Physalis* may consist of physalien and some other carotenoids (SIMPSON et al. 1978), could not be confirmed in our experiments. Only the isolation and chemical analysis of the small crystalloids can determine their nature.

The appearance of structures which pointed towards tubules glues together indicated the possibility of some kind of branching. On cross sections, sets of two, three, four or even more tubules glued together are clearly visible. The real nature of these structure is not yet known. It may be the result of the process of gluing two or more neighboring tubules, or maybe it is the site of formation of new tubules (PREBEG 2000). Past and present observations show that in the places of their formation (plastoglobules and membranes) only parallel bundles of tubules were presented (LJUBEŠIĆ 1977, WRISCHER et al. 1998).

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References

CAMARA, B., HUGUENEY, P., BOUVIER, F., KUNTZ, M., MONÉGER, R., 1995: Biochemistry and molecular biology of chromoplast development. Int. Rev. Cytol. 163, 175–247.

DERUÈRE, J., RÖMER, S., D'HARLINGUE, A., BACKHAUS, R. A., KUNTZ, M., CAMARA, B., 1994: Fibril assembly and carotenoid overaccumulation in chromoplasts: A model for supramolecular lipoprotein structures. Plant Cell 6, 119–133.

EMTER, O., FALK, H., SITTE, P., 1990: Specific carotenoids and proteins as prerequisites for chromoplast tubule formation. Protoplasma 157, 128–135.

HLOUŠEK-RADOJČIĆ, A., LJUBEŠIĆ, N., 1988: The development of daffodil chromoplasts in the presence of herbicides SAN 9789 and SAN 9785. Z. Naturf. 43c, 418–422.

KUHN, H., 1970: Chemismus, Struktur and Entstehung der Carotinkriställchen in der nebenkrone von *Narcissus poëticus* L.Var. »La Riante«. J. Ultrastruct. Res. 33, 332–355.

LAEMMLI, U. K., 1970: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680–685.

LICHTENTHALER, H. K., 1987: Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.

LJUBEŠIĆ, N., 1977: The formation of chromoplasts in fruits of *Cucurbita maxima* Duch. 'turbaniformis'. Bot. Gaz. 138, 286–290.

LJUBEŠIĆ, N., PREBEG, T., DEVIDÉ, Z., REGULA-BEVILACQUA, L., 1999: Chromoplasts in the sepals of *Physalis alkekengi*: The effect of norflurazon on chromoplast differentiation. Acta Bot. Croat. 58, 79–86.

LJUBEŠIĆ, N., WRISCHER, M., DEVIDÉ, Z., 1991: Chromoplasts – the last stages in plastid development. Int. J. Dev. Biol. 35, 251–258.

LJUBEŠIĆ, N., WRISCHER, M., DEVIDÉ, Z., 1995: Development of chromoplast tubules in *Hypericum* flowers. Period. Biol. 97, 333–336.

LJUBEŠIĆ, N., WRISCHER, M., DEVIDÉ, Z., 1996: Chromoplast structures in *Thunbergia* flowers. Protoplasma 193, 174–180.

PREBEG, T., 2000: Mehanizmi izgradnje lipoproteinskih struktura u kromoplastima (Formation of lipoprotein structures in chromoplasts). MSc Thesis, University of Zagreb.

SALOPEK, B., LJUBEŠIĆ, N., 1994: The fine structure of pepper chromoplasts: The effect of bleaching herbicides. Acta Bot. Croat. 53, 7–13.

SIMPSON, D. J., BAQAR, M. R., LEE, T. H., 1978: Chromoplast ultrastructure in fruit of *Solanum pseudocapsicum* and fruit and sepals of *Physalis alkekengi*. Aust. J. Bot. 26, 793–806.

SITTE, P., FALK, H., LIEDVOGEL, B., 1980: Chromoplasts. In: CZYGAN, F.-C. (ed), Pigments in plants. 2nd ed., 117–148. G. Fischer Verlag, Stuttgart.

VISHNEVETSKY, M., OVADIS, M., VAINSTEIN, A., 1999: Carotenoid sequestration in plants: the role of carotenoid-associated proteins. Trends Plant Sci. 4, 232–235.

WRISCHER, M., LJUBEŠIĆ N., DEVIDÉ, Z., 1998: The influence of norflurazon on the formation of chromoplast tubules in *Hypericum perforatum* flowers. Acta Bot. Croat. 57, 11–18.

WRISCHER, M., LJUBEŠIĆ, N., PREBEG, T., MAGNUS, V., 1999: The succession of chromoplast structures in *Impatiens noli-tangere* flowers. Phyton (Horn) 39, 49–59.

WRISCHER, M., PREBEG, T., MAGNUS, V., LJUBEŠIĆ, N., 2001: Ultrastructural study of chromoplast components rich in glycolipids. Acta Bot. Croat. 60, 141–147.