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Phyton (Horn, Austria)	**Vol. 39**	Fasc. 1	49–59	20. 8. 1999

The Succession of Chromoplast Structures in *Impatiens noli-tangere* Flowers

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With 9 Figures

Received October 20, 1998

Accepted November 30, 1998

Key words: *Impatiens noli-tangere* L., flower, differentiation of chromoplasts, ultrastructure

Summary

WRISCHER M., LJUBEŠIĆ N., PREBEG T. & MAGNUS V. 1999. The succession of chromoplast structures in *Impatiens noli-tangere* flowers – Phyton (Horn, Austria), 39 (1): 49–59, 9 figures. – English with German summary.

The differentiation of chromoplasts in the petals and sepals of *Impatiens noli-tangere* flowers was studied by ultrastructural, pigment and protein analyses. The differentiation of chromoplasts in both tissues started in the buds with young chloroplasts, which in their stroma, contained lightly stained globules (LSGs), 20 to 40 nm in diameter. In petal chromoplasts of open flowers these LSGs gradually disappeared and long tubules of 15 to 20 nm diameter developed, possibly by outgrowth from the LSGs. Later on the tubules arranged into tight bundles. In most chromoplasts of sepals the LSGs remained in the stroma throughout the life span of the flower, while the tubules appeared only in chromoplasts of some cell groups.

The sepals contained more carotenoids (0.564 mg/g fr. wt.) than petals (0.384 mg/g fr. wt.). On the other hand, the sepal tissue of open flowers was poorer in the characteristic tubular protein of 32–35 kD, having about 60% of that present in petal chromoplasts. These results indicated that the differentiation of sepal chromoplasts occurred at a slower rate and rarely proceeded beyond the LSG-stage.

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Zusammenfassung

WRISCHER M., LJUBEŠIĆ N., PREBEG T. & MAGNUS V. 1999. Die Abfolge der Chromoplastenstrukturen während der Entwicklung der Blüten von *Impatiens noli-tangere*. – Phyton (Horn, Austria) 39 (1): 49–59, 9 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Differenzierung der Chromoplasten in den Kelch- und Kronblättern der Blüten von *Impatiens noli-tangere* wurde mit Hilfe von Feinstruktur-, Pigment-, und Protein-Analysen untersucht. Die Knospen beider Gewebe enthielten junge Chloroplasten mit kleinen hellen Globuli (Durchmesser 20–40 nm) im Stroma. Nach Öffnung der Blüte verschwanden diese hellen Globuli allmählich aus den Chromoplasten der Kronblätter. Statt dessen erschienen lange Tubuli (Durchmesser 15–20 nm), die sich möglicherweise aus den hellen Globuli entwickelten. Die Tubuli ordneten sich später in enge Bündel. In den meisten Chromoplasten der Kelchblätter blieben die hellen Globuli während der ganzen Lebensdauer der Blüte erhalten; nur in vereinzelten Zellgruppen erschienen Tubuli in den Chromoplasten.

Die Kelchblätter enthielten mehr Carotinoide (0,564 mg/g Frischgew.) als die Kronblätter (0,384 mg/g Frischgew.). Das Gewebe der Kelchblätter der offenen Blüten enthielt jedoch etwa 40% weniger von dem für Tubuli charakteristischen Protein (32–35 kDa). Die gewonnenen Ergebnisse zeigen, daß die Differenzierung der Chromoplasten in den Kronblättern schneller verläuft als in den Kelchblättern, in denen das Stadium der hellen Globuli selten überschritten wird.

Introduction

The intense, yellow, orange or red, colours of many flowers and fruits originate from carotenoids that accumulate (in large quantities) in their chromoplasts. Carotenoids are always localized in special substructures. Which type of such substructures will develop appears to depend on the lipid-to-protein ratio synthesized during chromoplast differentiation (SITTE & al. 1980, DERUÈRE & al. 1994). High lipid levels induce the formation of globular inclusions (chromoplast plastoglobules), whereas high protein levels favour the formation of either chromoplast membranes or long filamentous structures, termed tubules or fibrils. Sometimes several types of structures develop simultaneously, or in succession, in the same chromoplast (KNOTH & al. 1986, LJUBEŠIĆ & al. 1996).

The tubular chromoplast type has been found in many flowers and fruits (CAMARA & al. 1995). It is claimed that the core of the tubules consists of carotenoids with strictly parallel oriented molecules arranged along with the length of the tubule. By polarizing light microscopy the tubules are strongly birefringent when aggregated into bundles. According to the models of the molecular organization of tubules published to date, the carotenoid core is enveloped either by a single layer composed of both lipids and proteins, or by two layers: an inner layer of lipids and a peripheral layer of proteins (KNOTH & al. 1986, DERUÈRE & al. 1994). While the set

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of carotenoids and lipids (mostly glyco- and phospholipids) found in tubules varied from species to species, a characteristic protein of 30–35 kDa, the s.c. fibrillin, was detected in all chromoplast tubules examined so far (WINKENBACH & al. 1976, WUTTKE 1976, SMIRRA & al. 1993, DERUÈRE & al. 1994, LJUBEŠIĆ & al. 1996).

Isolated tubules examined with the electron microscope using the negative staining method appear as compact threads. However, on thin sections of material fixed and stained with heavy metals (OsO_4, $KMnO_4$), the core of the tubules is always lightly stained and the periphery is dark. This could be explained by assuming that the carotenoid core cannot be preserved by the fixing agents and is thus dissolved during dehydration and embedding.

The chromoplasts in petals of *Impatiens noli-tangere* flowers have been classified as tubular chromoplasts (SITTE & al. 1980). We investigated the mode of formation of chromoplasts both in the petals and in the corollinic sepals (spurs) of these flowers. The pigment and protein analyses indicated the presence of similar constituents in both tissues, but in different concentrations. By electron microscopy we observed that there were differences between the two tissues in the speed of formation of chromoplast ultrastructures, those in the sepals remaining, throughout flower development, in a juvenile state.

Materials and Methods

Petals and sepals (spurs) of several developmental stages of buds and flowers of *Impatiens noli-tangere* L. were studied.

Hand made sections through fresh petal and sepal tissue of open flowers were examined with the light microscopes Zeiss Axiovert 35 and Axiolab.

For ultrastructural studies small pieces of tissues were fixed for 1 h in 1% glutaraldehyde in cacodylate buffer (pH 7.2). After rinsing in the same buffer, the material was postfixed for 2 h in 1% OsO_4 in the same buffer and, after dehydration, embedded in araldite. Thin sections of the material were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10A electron microscope.

Pigments from petals and sepals of open flowers were extracted with acetone. Carotenoids were separated by thin-layer chromatography on silica gel G plates developed with petrol : acetone (70 : 30), eluted from the respective zones with acetone and quantified spectrophotometrically.

For protein analyses, 1 g of petals or sepals (spurs) from open flowers was ground in a blender in 10 mM $MgCl_2$, 2.5% polyvinylpyrolidone, and 1 mM phenylmethylsulfonyl fluoride. After filtration, the homogenate was centrifuged at 10,000 g for 15 min, the sediments resuspended in the same buffer and used for electrophoresis. Proteins were determined after BRADFORD 1976. Proteins were separated in 12% SDS-PAGE (LAEMMLI 1970), stained with Coomassie brillant blue and analyzed by video densitometry using Master ™ VDS Soft, version 2.0 (Pharmacia Biotech. Inc.).

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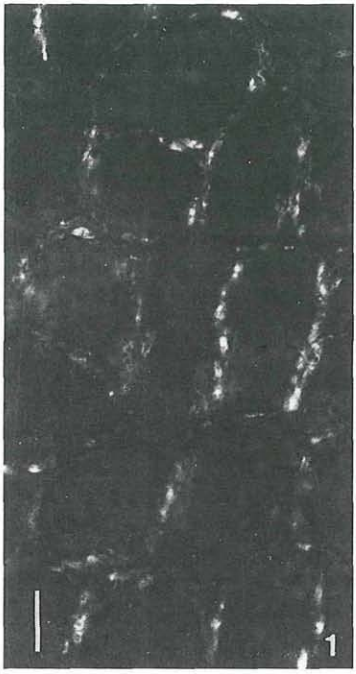

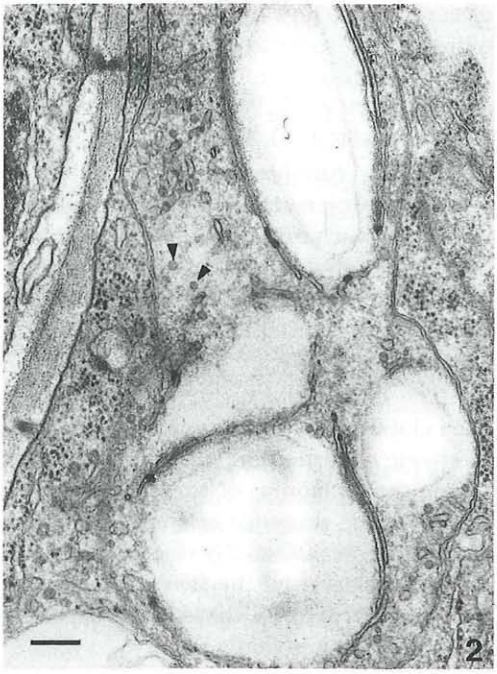

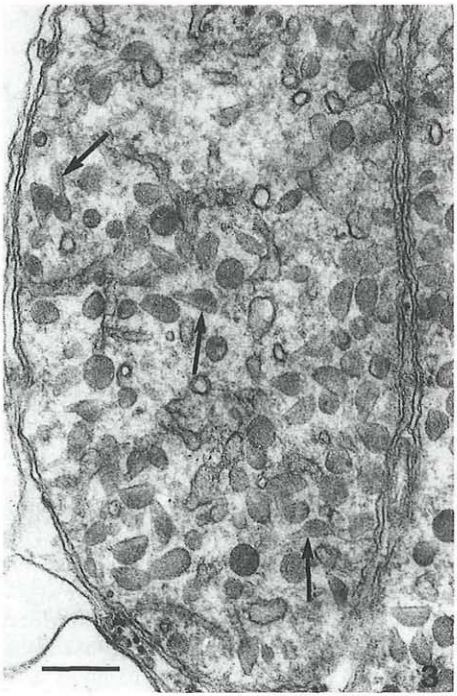

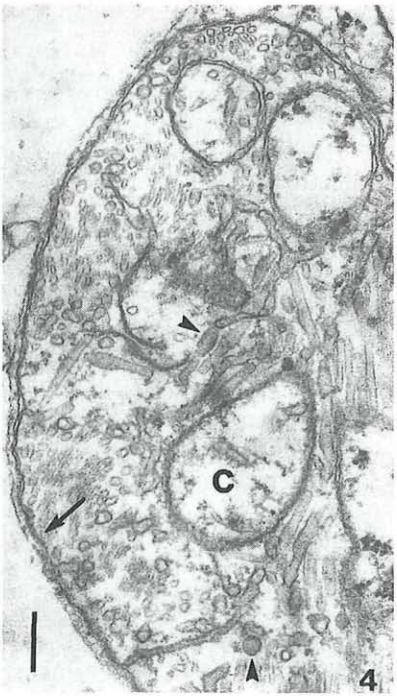

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Results

Both petals and sepals of very young buds (about 2.5 mm long) were light-green, and turned yellow-green in older (about 5 mm long) buds. In unfolding flowers the sepals were still yellow-green, while the petals became light-yellow. At anthesis, the colour of both petals and sepals was light-yellow.

Light microscope observations

The chromoplasts in petals at anthesis were spindle shaped or oval. They contained several intensely yellow coloured, elongated structures, which in the polarizing microscope were strongly birefringent (Fig. 1). In sepals the plastids were smaller than in petals, and generally did not show birefringence in the polarizing microscope. Only isolated groups of cells contained plastids with birefringent inclusions.

Ultrastructural analyses

Development of chromoplasts in petals

Petals of 2.5 and 5 mm long flower buds contained plastids with large starch grains. The thylakoid system in plastids of the mesophyll cells consisted of a few small grana, while in epidermal cells it was very reduced (Fig. 2). Dark droplets, consisting probably of lipids, were occasionally attached to the thylakoids. In both cell types there were also some irregularly shaped vesicles of the peripheral reticulum formed by invagination of the inner membrane of the plastid envelope. In the stroma, in addition to ribosomes, lightly stained globules (LSGs), 25 to 40 nm in diameter, similar to plastoglobules, were present. Some of these globules were homogeneously light gray, and some had a more lightly stained core. Plastids of 5 mm long buds contained numerous LSGs.

In the mesophyll cells of unfolding, 10 mm long flowers there were still chloroplasts with few grana of 3–5 thylakoids, while the thylakoid system

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Fig. 1. Petal tissue of *Impatiens noli-tangere* flowers under the polarizing light microscope. The birefringent structures represent the bundles of tubules in the chromoplasts. Bar = 10 μm.

Figs. 2–8. Electron microscopy of sections through chromoplasts of *Impatiens noli-tangere* flowers. LSG = lightly stained globule. Bars = 0.2 μm.

Fig. 2. Plastid from a flower bud with thylakoids, starch grains and small LSGs (arrowheads).

Fig. 3. Part of a petal chromoplast from unfolding flower. There are numerous LSGs, some with protruding tubules (arrows).

Fig. 4. Petal chromoplast from a 10 mm long, open flower with tubules of different diameters and LSGs (arrowheads). Contact of tubules with the envelope (arrow) and invaginations of the cytoplasm are indicated (c).

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of the epidermal cells was rudimentary. There also were numerous vesicles belonging to the peripheral reticulum and some starch grains. The stroma contained, in addition to some ribosomes, numerous large LSGs (30 to 60 nm in diameter). These were often somewhat elongated and in some of them electron-translucent portions appeared. In some places, short tubules were outgrowing from the LSGs, which then often attained a half moon shape (Fig. 3).

In the chromoplasts of further developed petals numerous long tubules appeared (Fig. 4). At first, the diameter of the tubules varied within wide limits. Some tubules were wide, with a diameter varying from 30 to 60 nm. The others, more numerous, were much thinner, very long and slightly bent. The diameter of these tubules was more uniform, and varied from 15 to 20 nm. Their outlines, as seen in cross-sections, were irregularly roundish. The true lengths of the tubules could not be determined, as they disappeared from the thin sections. The LSGs were still present in the stroma, and long tubules were found to be in contact with some of them. Some pictures also indicated a transit of broad tubules into narrow ones. A contact of tubules (both wide and narrow ones) with the plastid envelope was also noticed in some places (Fig. 4). Few short single thylakoids, some vesicles of the peripheral reticulum and starch grains were still present in the plastids, while ribosomes were scarce. The chromoplasts occasionally attained irregular, amoeboidal forms. Portions of the cytoplasm – sometimes including a mitochondrion – were found in plastid invaginations (Fig. 4).

In chromoplasts of open, 20 mm long flowers the thin tubules were usually arranged into bundles (Fig. 5, 7). Per section of a chromoplast there were often several bundles of tubules lying in different directions. The wide tubules were scarce. In the stroma there remained some LSGs, some vesicles of the peripheral reticulum, short segments of thylakoids and small starch grains.

In petals of flowers at anthesis, 30 mm long, the chromoplasts were considerably enlarged. Their stroma appeared to be empty, with only few membranes, vesicles and dark plastoglobules. There were some loose bundles of long tubules, but also single tubules lying in all directions in the stroma. The LSGs were scarce (Fig. 8). In the cytoplasm, close to the chromoplasts, large globular inclusions, probably lipidic, were occasionally found. Although the chromoplast fine structure looked somehow se-

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Fig. 5. Petal chromoplast from a 20 mm long flower with bundles of tubules (t), LSGs (arrowheads) and a starch grain.

Fig. 6. Part of a sepal chromoplast with LSGs and tubules of different diameters.

Fig. 7. Transversely cut bundle of tubules from a petal chromoplast.

Fig. 8. Part of a petal chromoplast from a flower at anthesis with scarce, loosely arranged tubules. A large lipid globule in the cytoplasm (g).

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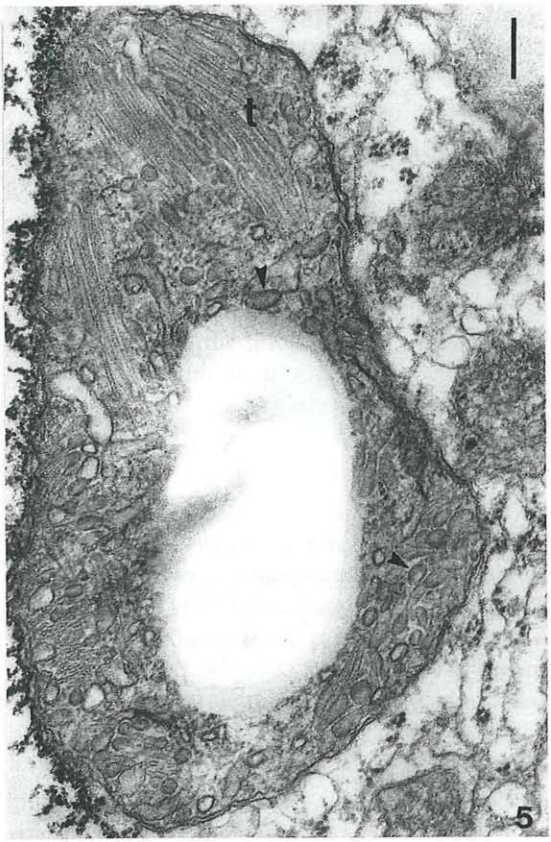

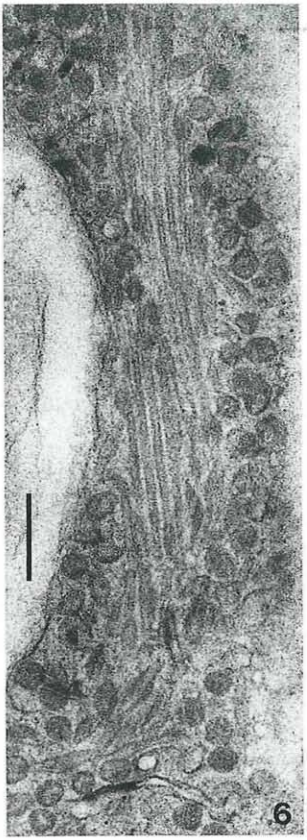

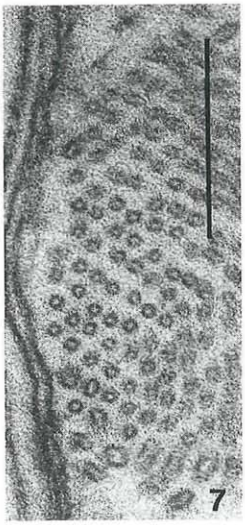

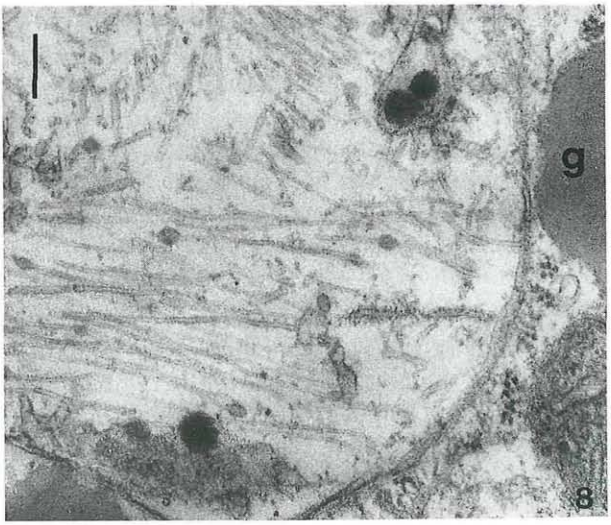

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nescent, that of other components of the cells, e.g. of the mitochondria, still appeared quite normal.

Development of chromoplasts in sepals (spurs).

The differentiation of sepal plastids in the buds proceeded similarly to that in the petals. The development started with chloroplasts containing numerous LSGs in the stroma, but the further differentiation of chromoplasts occurred more slowly than in the petals, so that in those of open, 20 mm long flowers there were still plastids with some thylakoids, but also with numerous LSGs in the stroma. However, in chromoplasts of some cell groups, in addition to LSGs, small bundles of long tubules were found (Fig. 6). In chromoplasts of the sepals at anthesis (in 30 mm long flowers), the LSGs were the main substructure of the stroma.

Pigment content

The pigments were extracted from petals and sepals at anthesis. The petals contained 0.384 mg/g of fresh weight of carotenoids, and the sepals 0.564 mg/g of fresh weight. Thin-layer chromatography revealed that the major pigments in the petals were acylated violaxanthin (47.1%) and lutein epoxide (38.1%). There also were small quantities of lutein and of several other carotenoids (mostly xanthophylls) (14.8%). From the sepals the same two major carotenoids were isolated: acylated violaxanthin (44.1%) and lutein epoxide (35.6%). The remaining 20.3% were lutein and several other minor carotenoids. In the sepals, traces of chrorophylls a and b were always present.

Protein content

Only petals and sepals at anthesis were available in quantities sufficient for protein analyses. SDS-PAGE indicated, both in the petals and in the sepals, the presence of a polypeptide with a molecular weight of 32–35 kDa (Fig. 9). Densitometric analyses of Coomassie blue stained gels indicated that the level of this protein in the sepals was only approximately 60% of that in the petals.

Discussion

Two structures are characteristic for chromoplasts of *Impatiens* petals: tubules and LSGs. While tubules prevailed in petals at anthesis, LSGs prevailed at the bud stage. During the unfolding of the petals the LSGs gradually disappeared, and the number of tubules increased drastically. In contrary to the situation in petals, in most sepal chromoplasts the LSGs prevailed at anthesis. There also were differences in chemical constituents between petals and sepals. Both contained the same set of pigments (ca-

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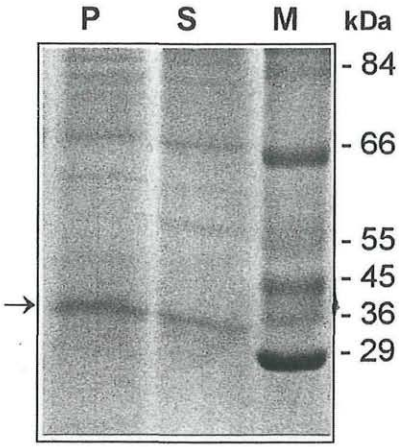

Fig. 9. SDS - PAGE of total proteins extracted from petals and sepals of *Impatiens noli-tangere* flowers. M = marker proteins, P = petals, S = sepals. The arrow indicates the 32–35 kDa polypeptide.

rotenoids), although their content was higher in sepals than in petals. On the other hand, although the characteristic tubular protein (32–35 kDa) was present in both tissues, its level in sepals reached about 60% of that in petals. According to these findings, it seems that only sufficiently high amounts of this protein would enable the formation of tubules. Experiments of EMTER & al. 1990, applying specific inhibitors on flowers containing tubular chromoplasts, confirmed that for the formation of tubules both pigments and the specific tubular protein were necessary. In vitro experiments performed by DERUÈRE & al. 1994 supported this statement as well. When comparing the ultrastructure of sepal chromoplasts with those found in bud tissue, we may conclude that in sepals the differentiation of tubular chromoplasts is slowed down, so that they remain in a juvenile state.

As observed on thin sections of petal chromoplasts of unfolding flowers, the LSGs seem to be the sites of formation of tubules, and possibly a "depot" of material necessary for their assembly. We suppose, that the LSGs contain the main components necessary for the formation of tubules. The role of chromoplast envelopes in the evolvement of tubules should also be taken into consideration. A contact of tubules with the chromoplast envelope was particularly frequently observed in young petals. It was already suggested, that, similarly to the situation in chloroplasts, the inner membrane of the chromoplast envelope should be active in the formation of different chromoplast substructures (CAMARA & al. 1995).

Lightly stained bodies of similar ultrastructure as LSGs were described in the chromoplasts of flowers of *Tropaeolum majus* (FALK 1976). In contrary to the LSGs in Impatiens, "isodiametric bodies" in *Tropaeolum*

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were not precursors of tubules, but appeared at a time when the tubules were already present in the stroma. Isolated "isodiametric bodies" contained proportionally more pigments and less proteins and lipids than the tubules (WINKENBACH & al. 1976). Both "isodiametric bodies" of *Tropaeolum* and LSGs of *Impatiens* flowers resemble the darkly stained, osmiophilic plastoglobules, that in many chromoplasts are known to play a role in the formation of tubules. Contacts of tubules with small osmiophilic lipid droplets have been observed in the chromoplasts – at various developmental stages – of several flowers and fruits (WUTTKE 1976, KNOTH & al. 1986, LJUBEŠIĆ & al. 1995). A direct outgrowth of the tubules from large plastoglobules is also known (SIMPSON & al. 1975, LJUBEŠIĆ 1977).

In *Impatiens* flowers, the differentiation of their chromoplasts starts with the appearance of LSGs and ends with the formation of tubules. How far this differentiation may go, seems to depend on the balance between the synthesis of pigments, tubular proteins and lipids (DERUÈRE & al. 1994). It is striking, that the life span of tubular chromoplasts in *Impatiens* flowers is short. Their senescence began at a time when other cell constituents still retained a normal ultrastructure and function. The senescence was expressed in a significant decrease in the number of tubules, a phenomenon already observed in other plants containing tubular chromoplasts (SMITH & BUTLER 1971, FALK 1976, LJUBEŠIĆ & al. 1995). SMIRRA & al. 1993 reported that, in cucumber flowers at anthesis, the content of tubular protein rapidly decreased to a very low level. At the time of the disappearance of tubules in *Impatiens* petal chromoplasts the colour of the tissue and the content of pigments were still unchanged. The question is to what structures the pigments may then be bound. These structures may be plastoglobules, but large lipid inclusions in the cytoplasm could not be excluded either.

Acknowledgement

The authors would like to thank B. SALOPEK-SONDI, M. Sc., for the protein analyses.

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Zeitschrift/Journal: Phyton, Annales Rei Botanicae, Horn

Jahr/Year: 1999

Band/Volume: 39_1

Autor(en)/Author(s): Wirscher Mercedez, Ljubesic Nikola, Preberg Tatjana, Magnus Volker

Artikel/Article: The Succession of Chromoplast Structures in Impatiens noli-tangere Flowers. 49-59