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

# Ultrastructural study of chromoplast components rich in glycolipids

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A cytochemical method for the ultrastructural localization of glycolipids, performed on thin sections, was used in the study of tubular chromoplasts. The method gave clear pictures of structures that contained glycolipids, while other chemical constituents were not stained. The method was tested on two flowers (*Impatients noli-tangere* L. and *Thunbergia alata* Boj. ex Sims) containing different types of tubular chromoplasts. The ultrastructure and mode of development of the two types of tubules are discussed and compared with the results obtained by standard staining methods.

**Key words:** *Impatiens noli-tangere*, *Thunbergia alata*, chromoplast tubule, ultrastructure, glycolipid staining

**Abbreviations:** TCH-Ag proteinate = thiocarbohydrazide-silver proteinate, TCH-$OsO_4$ = thiocarbohydrazide-osmium tetroxide

## Introduction

The large quantities of carotenoids, which accumulate in the chromoplasts of many yellow and red flowers and fruits, are localized in different submicroscopic structures (SITTE et al. 1980, LJUBEŠIĆ et al. 1991, CAMARA et al. 1995). One type of these structures, the tubules, are of particular interest for ultrastructural research because they contain, in addition to carotenoids, lipids and proteins organized in a specific pattern (VISHNEVETSKY et al. 1999). For better visualization of the tubules, but also of other lipid-rich structures in chromoplasts, a cytochemical method for ultrastructural localization of glycolipids (mono- and digalactosyldiacylglycerols) was introduced (DERUÈRE et al. 1994). This staining method afforded much more informative electron micrographs of chromoplasts than standard staining procedures, thus providing new insights into the ultrastructure of chromoplast tubules and their interaction with other chromoplast structures. The results of these comparative studies are reported herein.

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## Materials and Methods

The cytochemical methods used are based on the oxidation of the sugar residues in glycolipids using periodic acid. The aldehyde groups thus formed are reacted with thiocarbohydrazide (TCH) and the resulting hydrazones are effective chelators for heavy metals, such as silver or osmium. The site containing such metal complexes can then be detected in the electron microscope.

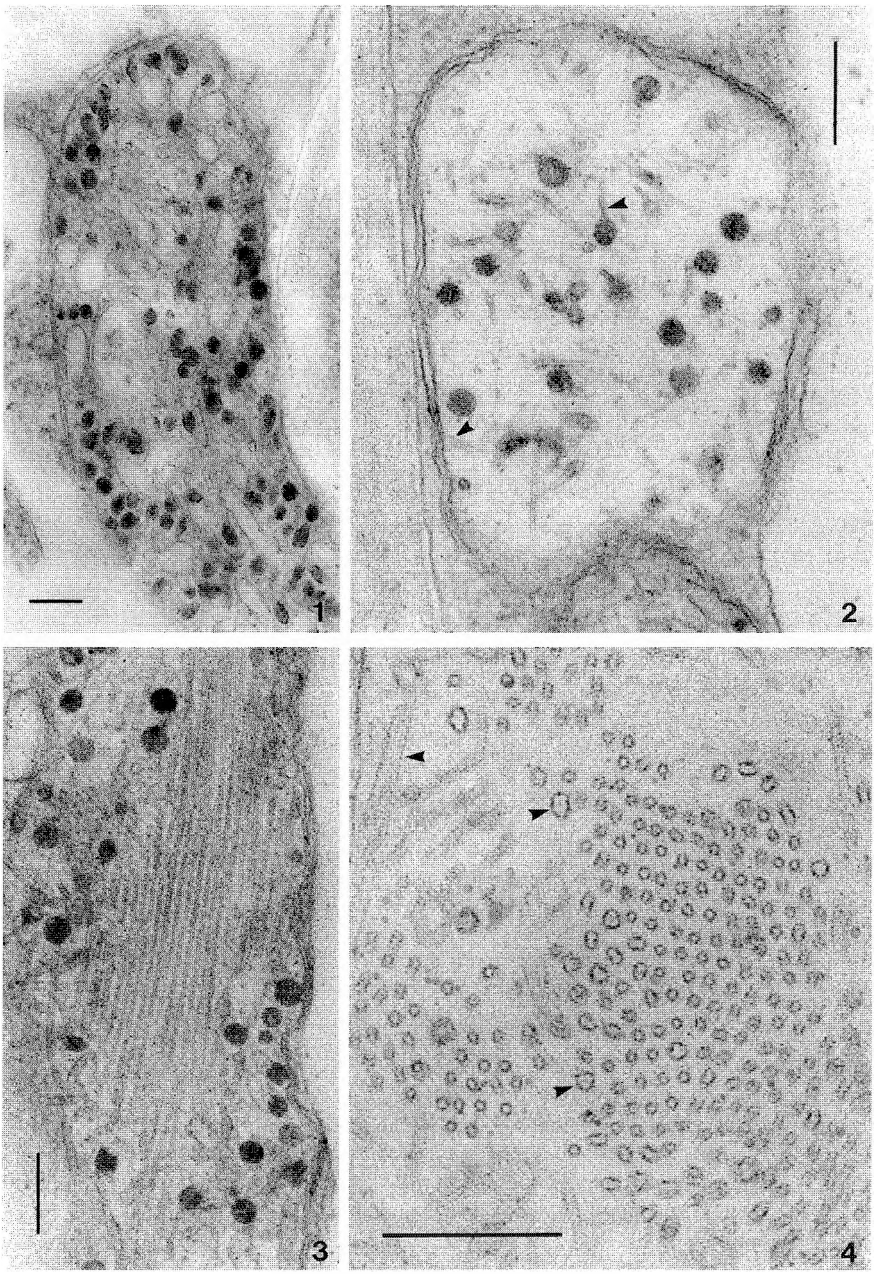
The method was tested on two plants containing flowers with different types of tubular chromoplasts (LJUBEŠIĆ et al. 1996, WRISCHER et al. 1999). The tissue of buds and open flowers of *Impatiens noli-tangere* L. and *Thunbergia alata* Boj. ex Sims was prepared using standard fixation and embedding methods (WRISCHER et al. 1999). Pieces of tissue were fixed using 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h. After washing in buffer the tissue was postfixed in 1% $OsO_4$ in the same buffer for 2 h and, after dehydration, embedded in araldite or in Spurr's medium. The sections used as controls were stained with uranyl acetate and lead citrate, those used for visualization of the carbohydrate components of glycolipids remained unstained.

For the visualization of glycolipids a method described by THIÉRY (1967) and LEWIS and KNIGHT (1977) was used with slight modifications. Thin sections were first floated for 45 min on the surface of an 1% solution of periodic acid in distilled water and, after rinsing with several changes of distilled water, were treated for 30 min with 0.2% thiocarbohydrazide (TCH) in 20% acetic acid. They were then transferred to the surface of 10% acetic acid (two changes, each for 10 min), followed successively by 5% acetic acid (10 min), 1% acetic acid (10 min), and three changes of double distilled water (each for 10 min). The sections were then kept for 30 min on the surface of 1% silver proteinate (Silver protein, mild, Sigma) in double-distilled water. After washing with several changes of double-distilled water, the sections were mounted on carbon coated grids and dried. In a modified protocol, the sections were, instead of the silver proteinate treatment, mounted on carbon-coated grids and kept for 1 h on drops of 2% $OsO_4$ (at 50 °C), then rinsed with distilled water and dried. The stained sections were examined in the electron microscope Zeiss EM 10A.

## Results

Both the TCH-Ag proteinate and the TCH-$OsO_4$ methods gave similar results. When the sections were treated with periodic acid, the staining (i.e. contrast) of all cell components disappeared. When subsequently they were processed by the TCH-Ag proteinate or the TCH-$OsO_4$ protocols, only glycolipids were stained. Extraordinary clear electron micrographs of the glycolipid-containing structures thus resulted. The TCH-Ag proteinate procedure afforded particularly well-resolved pictures.

Both *Impatiens* and *Thunbergia* flowers contained tubular chromoplasts, although there were structural and developmental differences. In *Impatiens* flowers, the first carotenoid-containing structures that appeared in the buds were numerous plastoglobules of 30–60 nm diameter. They were strongly stained by the glycolipid staining procedure (Fig. 1). The tubules appeared somewhat later and were numerous in the chromoplasts of unfolding petals. Very often the tubules were found attached to plastoglobules, like outgrowing from them (Fig. 2). The tubules were also found in contact with the inner membrane of the




**Figs. 1–4.** Chromoplasts from *Impatiens noli-tangere* petals. 1 -TCH-$OsO_4$-staining; 2 – 4 -TCH-Ag proteinate-staining. Bars = 0.2 μm. 1 -Dark stained plastoglobules in bud chromoplasts; 2 -Chromoplast from a flower at anthesis. The contacts of tubules with plastoglobules and with the inner membrane of the envelope are indicated (arrows); 3 -Parallelly arranged tubules and dark plastoglobules in the chromoplast of an open flower; 4 -Cross- and longitudinally-sectioned tubules in the chromoplast of an open flower. Arrowheads indicate stacked tubules.

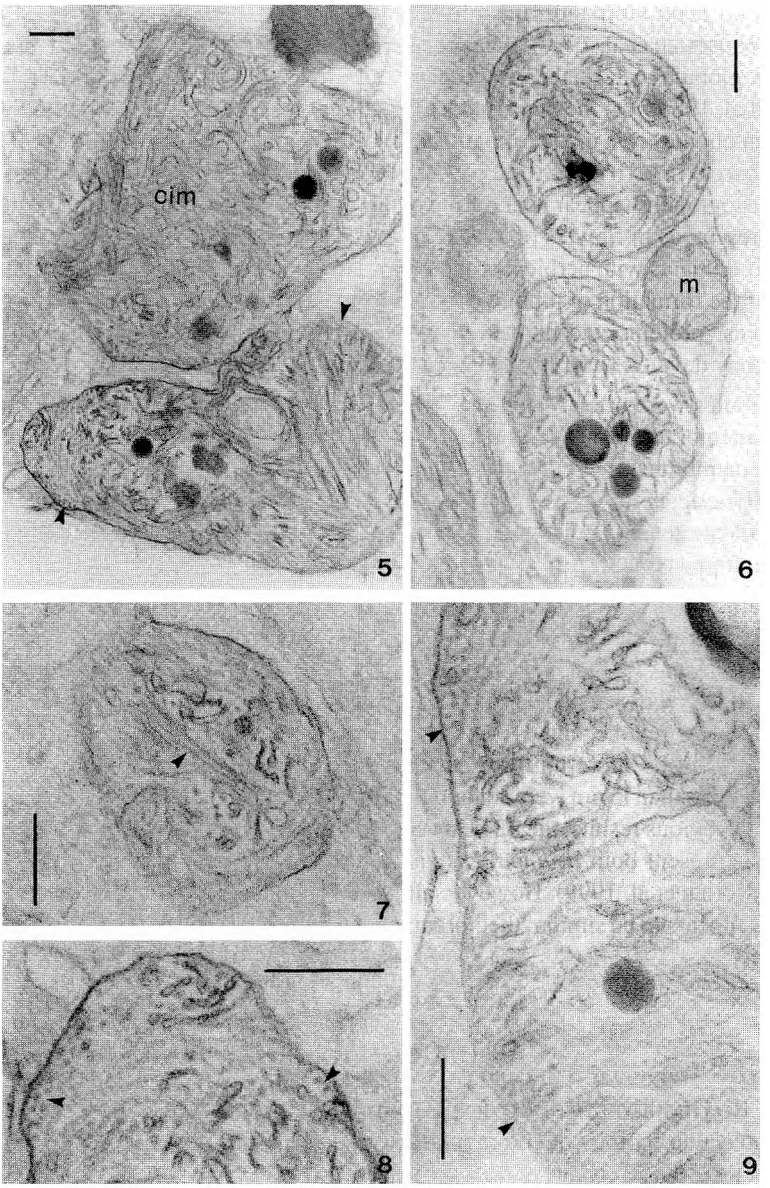
chromoplast envelope, indicating an alternative possible site of formation. The tubules were several μm long and were frequently arranged into groups oriented along the long axis of the chromoplast (Fig. 3). Individual tubules were straight or slightly wavy. At first, the diameter varied somewhat along the length of the tubule. Later, in flowers at anthesis, the diameter of the tubules became more uniform. In cross-sections, the tubules were roundish with an outer diameter varying from 15 to 20 nm. After glycolipid staining, the periphery of a tubule appeared as a dark granular ring 4 nm thick, while the core (7–10 nm) was more lightly stained. Sometimes two to several tubules were stacked together, a feature best seen when cross-sectioned. The outlines of these stacked tubules were larger and oval or irregularly shaped (Fig. 4).

In *Thunbergia* chromoplasts, the tubules appeared towards the end of chromoplast differentiation, i.e. in unfolding and open flowers. The tubules were particularly numerous in the chromoplasts of the upper epidermal cells, and were detected concomitantly with the disappearance of extended endomembranes from the chromoplast stroma (Fig. 5). In the plastids of the mesophyll cells the tubules gradually replaced the disintegrating thylakoids. The tubules were several μm long, rigid and thin (Fig. 7). After the glycolipid-staining, their cross-section could be better visualized than after standard preparations. Their overall diameter in cross-section was only 12–14 nm, and that of the lightly stained core about 8 nm (Fig. 8). Although some dark plastoglobules were present in the chromoplast stroma, a contact of the tubules with them was not detected (Fig. 6). On the other hand, contacts of the tubules with the envelope membrane were frequently found (Fig. 9).

## Discussion

It is known that for the formation of chromoplast tubules three components are necessary: bicyclic carotenoids, lipids (glyco- and phospholipids) and proteins (EMTER et al. 1990, DERUÈRE et al. 1994). Two tentative models have been proposed to explain the organization of chromoplast tubules. According to the first model, the core (wick) of the tubule contains carotenoid molecules oriented lengthwise in a regular fashion. The envelope would then be composed of an inner monolayer of lipids, and an outer layer of a 30–35 kDa protein called fibrillin (DERUÈRE et al. 1994). According to the second model the carotenoid-containing core is enveloped by a single layer which contains both lipids and globular 32 kDa proteins (KNOTH et al. 1986). After the TCH-Ag proteinate- or the TCH-$OsO_4$-staining only the glycolipids, but not the protein components of the tubular envelope were visualized. The dimensions of the tubules when cross-sectioned were similar to those found in standardly stained sections (LJUBEŠIĆ et al. 1996, WRISCHER et al. 1999). The core of the tubules was never stained. It is known that carotenoids are solubilized and removed during dehydration of the tissue. The stacking of tubules into groups of two or more, found in *Impatiens* chromoplasts appears to be a common phenomenon. The same pattern was, for example, observed in the fruit chromoplasts of *Solanum capsicastrum* (LJUBEŠIĆ et al. 2001) and in the flower chromoplasts of *Chelidonium majus* and *Agrimonia eupatoria* (PREBEG 2000).

It is known that the plastoglobules play a role in the formation of chromoplast tubules. In some chromoplasts the contact of the tubules with plastoglobules was obvious, and even their outgrowth from the globules could be clearly seen, as for instance in the chromoplasts




**Figs. 5–9.** Chromoplasts from *Thunbergia alata* petals. TCH-Ag proteinate-staining. Bars = 0.2 µm. 5 -Chromoplasts from a flower just before anthesis. The upper chromoplast is filled with »chromoplast internal membranes« (cim), the lower one contains bundles of tubules (arrowheads); 6 -Two chromoplasts from an open flower. The bundles of tubules are cut at different angles; large plastoglobules are seen in the stroma. m = mitochondrion; 7 -Chromoplast from an open flower. A longitudinally cut bundle of tubules is indicated (arrowhead); 8 -Part of a chromoplast from an open flower. The cross-sectioned tubules are indicated (arrowheads); 9 -Part of a chromoplast from an open flower. Some tubules are in contact with the inner membrane of the envelope (arrowheads).

of *Cucurbita pepo* »turbaniformis« fruit (LJUBEŠIĆ 1977) and in *Impatiens noli-tangere* flowers (WRISCHER et al. 1999). It has been proposed that carotenoids are first incorporated into plastoglobules, and later grow out into the stroma forming the core of the tubule (KNOTH et al. 1986). On the other hand, there are chromoplasts in which plastoglobules do not play an obvious role in the formation of the tubules, for example in flowers of *Cucumis* and *Thunbergia* (SMITH and BUTLER 1971, LJUBEŠIĆ et al. 1996). In a third type of chromoplasts, plastoglobules appear later during chromoplast differentiation, when the tubules are already present, as in flowers of *Tropaeolum* and *Hypericum* (FALK 1975, LJUBEŠIĆ et al. 1995). Experiments with herbicides and inhibitors of protein or carotenoid biosynthesis indicate that the formation of tubules depends on the balanced synthesis of all three components: lipids, proteins and carotenoids. When the synthesis of lipids prevails, plastoglobules may develop (EMTER et al. 1990, DERUÈRE et al. 1994).

The tubules were often found attached to the endomembranes and to the chromoplast envelope membrane, but the role of these membranes in the formation of tubules is not clear. The membranes may serve as the growing site (anchor) of the tubules. In the chromoplasts of *Hypericum* flowers, whose differentiation was partially inhibited by norflurazon – a blocker of carotenoid biosynthesis – very short, rudimentary tubules were found attached to the envelope membrane (WRISCHER et al. 1998).

The variation in the diameter of the tubules, as observed in *Impatiens* and in *Thunbergia* chromoplasts, cannot be explained at this point. In most tubular chromoplasts, »broad« tubules of 15–25 nm were found (CAMARA et al. 1995). WUTTKE (1976) reported that the thickness of the tubules in the chromoplasts of *Rosa rugosa* hips may even vary from 12 to 40 nm. It appears that the tubules become narrower toward the end of chromoplast differentiation, probably by more uniform packing of their components. Tubules of a uniform thickness of less than 15 nm were so far only described in *Thunbergia* chromoplasts. According to our previous results, the 32 kDa »carotenoid-associated protein« (VISHNEVETSKY et al. 1999) is present both in *Impatiens* and in *Thunbergia* chromoplasts (LJUBEŠIĆ et al. 1996, WRISCHER et al. 1999). However, although the protein composition of the two types of tubules appears to be similar, their organization at the molecular level may follow different patterns.

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