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THE INFLUENCE OF NORFLURAZON ON THE FORMATION OF CHROMOPLAST TUBULES IN *HYPERICUM PERFORATUM* FLOWERS

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The influence of norflurazon on the formation of the chromoplasts of *Hypericum perforatum* petals was studied by electron microscopy. Norflurazon (2×10^{-4} M) inhibited the synthesis of coloured carotenoids, impairing the assembly of chromoplast tubules and causing the accumulation of numerous small plastoglobules. The results were compared with the changes observed in the chromoplasts of control petals during maturation.

Key words: *Hypericum perforatum* L., petal, chromoplast differentiation, chromoplast tubule, norflurazon, electron microscopy

Introduction

A distinctive feature of many flowers and fruits is their intense yellow, orange or red colour, a consequence of coloured (cyclic) carotenoids accumulation in chromoplasts. Depending on the carotenoid-bearing structures, chromoplasts can be classified into several groups (SITTE et al. 1980, CAMARA et al. 1995). One group is the s.c. tubulous or fibrillar type present in many flowers and fruits. In these chromoplasts the pigments are located inside long filamentous lipoprotein structures, 15 – 20 nm in diameter, termed tubules or fibrils in the literature. In this paper, the term tubules will be used. It is supposed that pigments (coloured carotenoids) occupy the core of the tubule and are enveloped by lipids and pro-

teins, arranged either in one lipoprotein layer (KNOTH et al. 1986) or in two layers with the proteins arranged on the periphery (DERUÈRE et al. 1994). A protein of about 30–35 kDa, named fibrillin by DERUÈRE et al. (1994), appears to be the main protein in the tubules.

There are at least two ways for tubules to develop in chromoplasts. The tubules can develop by outgrowth from some globular lipid-containing structures (SIMPSON 1975, LJUBEŠIĆ 1977, WRISCHER et al. 1999). Alternatively, the tubules are first attached to plastid membranes, in particular, to the inner membrane of the plastid envelope. The role of these membranes in the formation of the tubules is not completely understood. This type of formation of tubules was described for the chromoplasts of a number of flowers (SMITH and BUTLER 1971, FALK 1976, LJUBEŠIĆ et al. 1995, 1996).

Experiments performed *in vitro* indicated that for the formation of tubules all three components – pigments, lipids and proteins – are necessary (DERUÈRE et al. 1994). Similar conclusions were derived from experiments with inhibitors (EMTER et al. 1990). A particularly specific inhibitor is the herbicide norflurazon (NF), which inhibits the enzyme phytoene desaturase and thus blocks carotenoid biosynthesis between phytoene and zeta-carotene (BÖGER 1996).

We studied the influence of NF on the formation of tubulous chromoplasts in *Hypericum perforatum* flowers. In an earlier study we confirmed that the petals of this species contained chromoplasts of the tubulous type (LJUBEŠIĆ et al. 1995).

Materials and methods

Flower buds of *Hypericum perforatum* L. were dipped for 24 h into an aqueous 2×10^{-5} M or 2×10^{-4} M solution of norflurazon (NF). Control buds were dipped into water for the same time.

For ultrastructural analysis, petals from buds and from open, control and NF treated flowers were used. Pieces of tissue were fixed in 1 % glutaraldehyde in cacodylate buffer (pH 7.2). After washing in buffer, the tissue was postfixed in 1 % OsO_4 in the same buffer, thoroughly dehydrated and embedded in araldite. Thin sections were stained with uranyl acetate and lead citrate and examined by the electron microscope Zeiss EM 10A.

Pigments were analysed by thin layer chromatography on silica gel G (acetone : petrolether = 30 : 70). For spectrophotometric analyses the pigments were extracted with 80 % acetone, identified spectrophotometrically and quantitatively determined according to Lichtenthaler (1987).

For protein analyses, 1 g of petals 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 were resuspended in the same buffer and used for electrophoresis. Proteins were determined after BRADFORD (1976). Proteins were separated in 12 % SDS-PAGE (LAEMMLI 1970), and stained with Coomassie brilliant blue.

Results

In young control buds the petals were light-green, changing their colour to yellow-green and yellow, at anthesis. The light-green tissue in the buds contained chloro-amyloplasts with small grana of 3–5 thylakoids, and numerous ribosomes in the stroma, accompanied by one to several large starch grains (Fig. 1). During further bud development the thylakoid system and the ribosomes progressively disappeared. The starch grains still remained, although they decreased in size and assumed irregular shapes. At this time, straight or slightly bent tubules appeared in the plastids, in contact with some internal membranes and with the inner membrane of the plastid envelope. The tubules were arranged into loose bundles (Figs. 2, 3, 4). These bundles were several μm long, although the true length of a single tubule could not be detected, as it usually disappeared from the thin section. The tubules were irregularly roundish in cross-section, with a lightly stained core and a dark (osmiophilic) rim (Fig. 3). This picture is the consequence of the fact that carotenoids cannot be fixed, and are dissolved during dehydration and embedding, resulting in a lightly stained core inside the tubule. The diameter of the tubules varied from 13–20 nm. In chromoplasts just before anthesis, darkly stained, droplet-like globules, 30–60 nm in diameter, became attached to some tubules. According to their shape, these globules may be concluded to have a fluid consistency (Fig. 5). After anthesis the chromoplasts enlarged and assumed irregular shapes. The not numerous tubules were now dispersed in an empty stroma. The dark globules, attached to the tubules, were also scarce (Fig. 6). Although these chromoplasts looked somehow senescent, other cell constituents, particularly the mitochondria, still had a normal ultrastructure.

No changes in colour and ultrastructure, were observed in petals treated with a 2×10^{-5} M solution of norflurazon (NF). The higher concentration of NF (2×10^{-4} M) caused conspicuous changes. While control buds reached anthesis within about 4 days, those treated with NF (2×10^{-4} M) required 7–8 days. There also were noticeable changes in the coloration of the NF-treated petals. Due to the twisted arrangement of the petals in the buds, the effect of NF-treatment was not uniform. Usually one half of the petal was more damaged and was completely white, while the other half was less damaged and yellow-white.

The plastids in NF-treated tissue were large and had irregular outlines. Starch was still present. In some plastids of yellow-white tissue, short tubules attached to the plastid envelope protruded into the stroma (Fig. 7). In other chromoplasts of the yellow-white tissue, short tubules were irregularly dispersed in the stroma. Dark (osmiophilic) globules (about 50 nm diameter) were observed in between, or attached to the tubules (Fig. 8). In white petals the plastids contained only numerous osmiophilic globules 40–90 nm in diameter (Figs. 9, 10).

Due to the very small quantities of tissue, pigment or protein analyses were not possible in the case of NF-damaged petals. Only petals of control flowers were available in quantities sufficient for such analyses. Thin layer chromatography showed that the predominant pigment in control petals was a carotene preliminary identified, by its R_f value, as zeta-carotene. Beta-carotene was present in much lower quantity, and xanthophylls could not be detected. This kind of pigment content in petals was similar to that found in some other tubulous

chromoplasts (WRISCHER at al. 1999). Protein analysis indicated the presence of polypeptides with the molecular weight reported for tubular proteins (DERUÈRE et al. 1994) of 30–35 kDa.

Discussion

Results obtained from both control and from norflurazon-treated petals confirmed the importance of the internal plastid membranes and the plastid envelope in the formation of tubules in *Hypericum* flowers. How the tubules assemble (grow) is not known. Experiments of their reconstruction *in vitro* have shown that a precise ratio of cyclic carotenoids, polar lipids (galactolipids and phospholipids), as well as of protein (fibrillin) is necessary (DERUÈRE et al. 1994). The decrease in the concentration of tubules in untreated flowers at anthesis is significant, and suggests a cessation of the synthesis of at least one of the compounds of the tubules.

The main effect of norflurazon (NF) is the blocking of the synthesis of cyclic (coloured) carotenoids (BÖGER 1996). As reported by EMTER et al. (1990), changes in the synthesis of coloured (cyclic) carotenoids can prevent the assembly of chromoplast tubules. This is clearly visible in NF-treated *Hypericum* petals with partial or total loss of coloration and assembly of the tubules. Recently it was reported that NF also influences the synthesis of chloroplast lipids (ABROUS et al. 1998). Norflurazon therefore may cause structural and functional changes in membranes and other structures that contain lipids. It should be mentioned that the plastid envelope can synthesize, and also contains, some carotenoids (JOYARD et al. 1998), and should therefore be particularly susceptible to the NF treatment.

In white, NF-damaged, petals, the tubules did not assemble and only globules of different diameters remained in the plastids. Plastoglobules are the most stable and most primitive form of plastid inclusions. They are found in all plastid types, from proplastids to senescent chloroplasts, as well as in chromoplasts. Plastoglobules represent accumulations of excess lipids, but may also contain

Figs. 1–6. Development of chromoplasts in control *Hypericum perforatum* petals (flowers).

Fig. 1. Part of a chloro-amyloplast from a young bud with small grana (g) and starch (s). 35,000 ×.

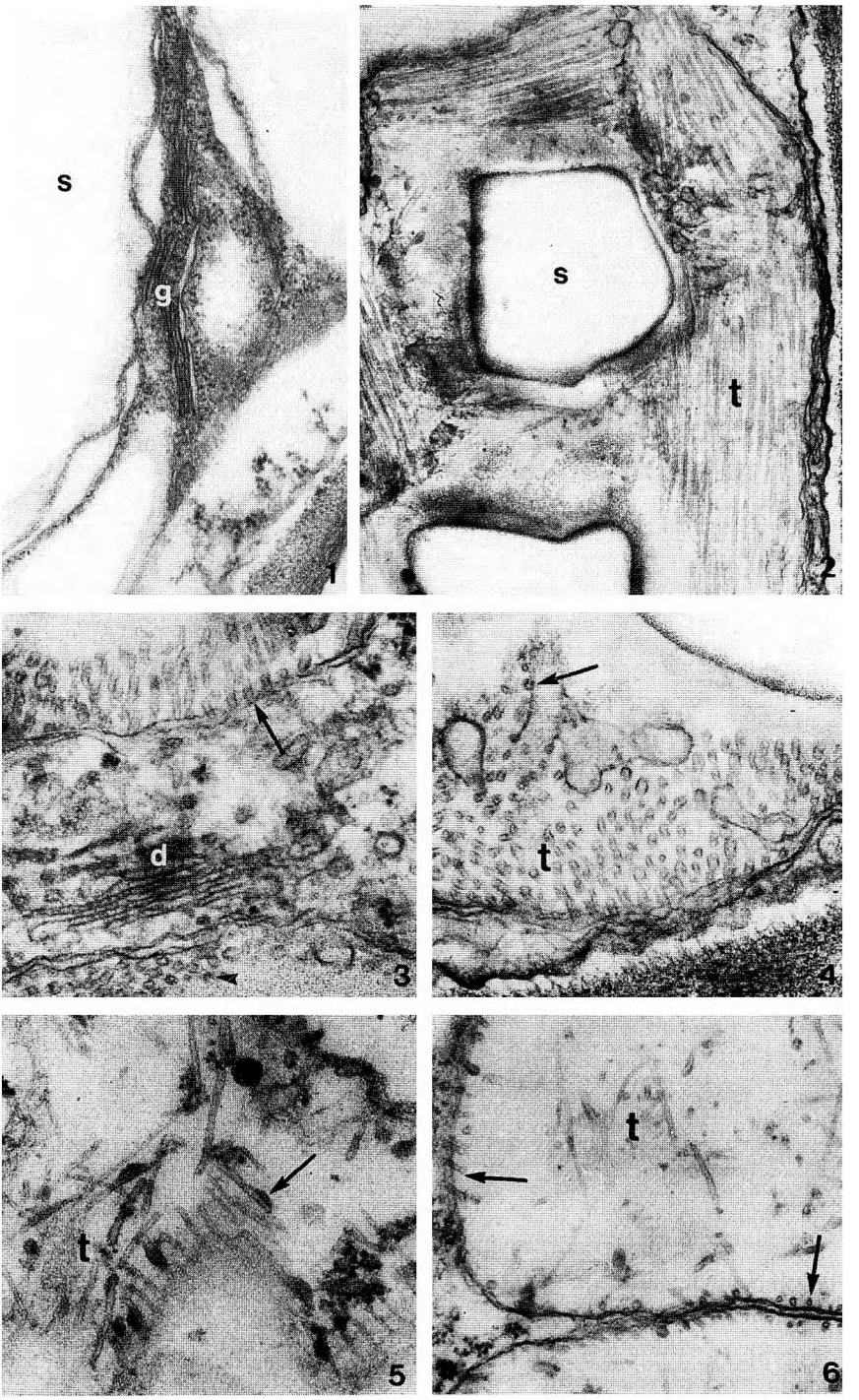
Fig. 2. Chromoplast from a bud with bundles of tubules (t) and starch (s). 34,000 ×.

Fig. 3. Parts of chromoplasts from a bud. In one chromoplast the tubules are attached to the plastid envelope (arrow), in the other the tubules are cross-sectioned (arrowhead). Dictyosom (d) is present in the cytoplasm. 60,000 ×.

Fig. 4. Part of a chromoplast from a bud. Tubules (t) are attached to the envelope. Some of the tubules are also attached to an inner membrane (arrow). 61,000 ×.

Fig. 5. Part of a chromoplast from a bud just before anthesis. The tubules (t) are irregularly arranged. Dark globules (lipid droplets) are attached to them (arrow). 53,000 ×.

Fig. 6. Part of a chromoplast from a petal at anthesis, with rare (sparse), irregularly arranged tubules (t). Some tubules are attached to the plastid envelope (arrows). 41,000 ×.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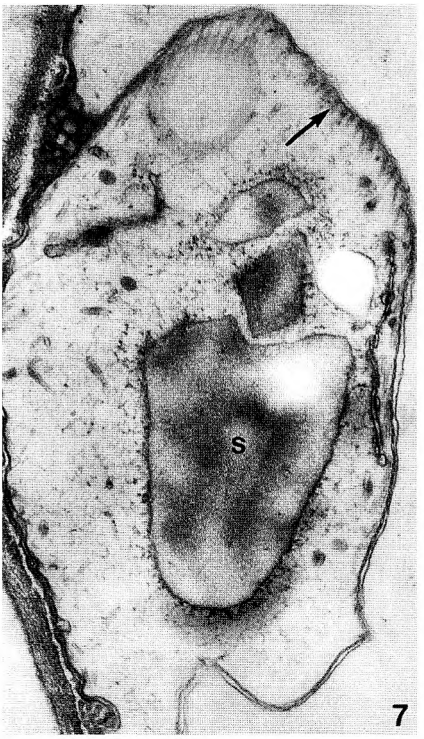
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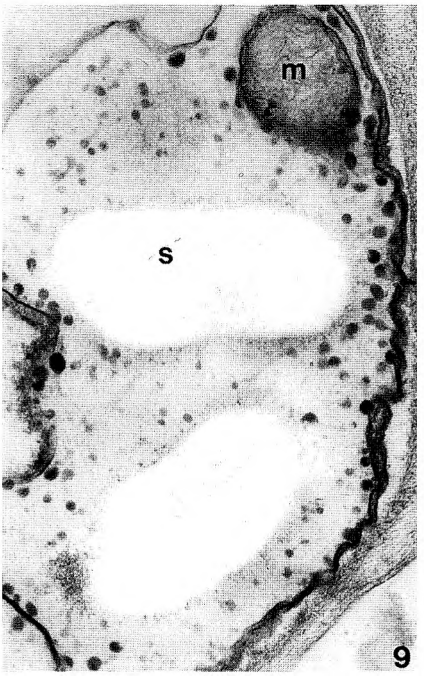
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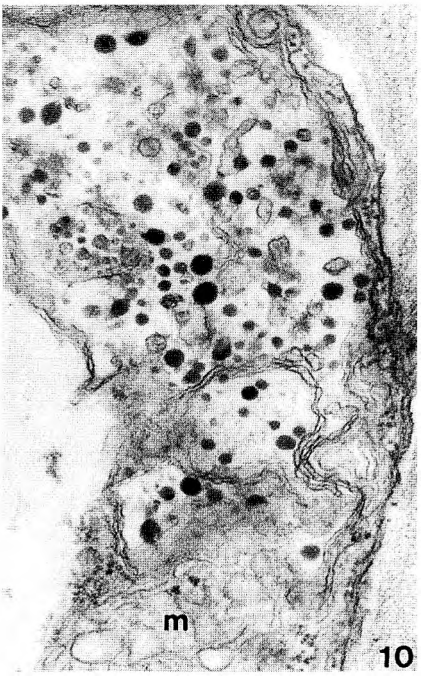
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Figs. 7-10. Chromoplasts in petals treated with 2×10^{-4} M norflurazon.

Fig. 7. Chromoplast from a yellow-white petal with short tubules (arrow) attached to the plastid envelope. Starch grains (s) are present in the stroma. 26,000 ×.

Fig. 8. Irregularly shaped chromoplasts from a yellow-white petal with tubules dispersed among small osmiophilic plastoglobules. 30,000 ×.

Fig. 9. Plastid from a white petal with small plastoglobules and starch grains (s). A mitochondrion (m) is lying in a plastid invagination. 31,000 ×.

Fig. 10. Plastid from a white petal with numerous osmiophilic plastoglobules of different diameter. A mitochondrion (m) with a normal ultrastructure is situated near the plastid. 44,000 ×.

lipid-soluble compounds, such as carotenoids in globulous chromoplasts (SITTE et al. 1980, LJUBEŠIĆ et al. 1991). It is probable, that the plastoglobules (lipid droplets) that accumulated in control *Hypericum* petals at anthesis, and those in NF-treated petals, have a different composition. The globules of control petals probably contain dissolved coloured carotenoids, while those in plastids of white NF-damaged tissue are obviously without coloured pigmentation. In addition, the lipid constituents may also be different.

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