Hormone Induced Changes in Carotenoid Composition in *Ricinus* Cell Cultures. II. Accumulation of Rhodoxanthin during Auxin-Controlled Chromoplast Differentiation

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The effects of auxin and cytokinin on light dependent carotenoid synthesis were studied in callus cultures derived from *Ricinus* endosperm tissue. Chloroplasts differentiate when calli are grown in the light in the presence of cytokinin and auxin. When cultures are transferred to an auxin-free medium, chromoplast differentiation is initiated, i.e. rhodoxanthin accumulates whereas lutein and chlorophyll content decrease and the plastid morphology changes from that of a typical chloroplast to that of a globular-type chromoplast. These changes are fully reverted by readdition of auxin. Plastid differentiation into either chloroplasts or chromoplasts in *Ricinus* endosperm cultures therefore appears to be controlled by auxin and cytokinin.

Introduction

Chromoplasts are yellow or red plastids generally found in fruits, flowers, and roots of higher plants. They can develop from any type of plastids but usually, for example, in ripening fruits or senescing leaves (for review see Thomson and Whatley [1]), they are derived from chloroplasts. The two types can be distinguished from each other not only by their fine structure but also by their pigment composition. While the photosynthetically active chloroplast contains chlorophylls and a fairly constant carotenoid pattern composed of β-carotene, lutein, violaxanthin, and neoxanthin, the photosynthetically inactive chromoplast is devoid of chlorophylls and accumulates a different set of carotenoids [2]. Thus, carotenoids may be a useful marker for studies on plastid differentiation.

In spite of the rather detailed knowledge of the structural and biochemical features of chromoplasts [2, 3], it is amazing how little we know about the regulation of plastid interconversion. One reason for our ignorance might be the complexity of the plastid-containing tissues of the intact plant. Cells in culture should provide a suitable material for the study of plastid development because growth conditions are defined and easy to manipulate.

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Here, we use a callus culture derived from *Ricinus* endosperm. The cells of this culture contain more or less differentiated plastids of a pregranal type (*c.f.* [1]) when grown in the dark. When transferred to light, some of these cells turned green due to the formation of chloroplasts. These cells had been used to establish a green strain (strain A, [4]) which forms red cells when transferred from an auxin-containing medium to an auxin-free medium. In a recent report we identified the red pigment as rhodoxanthin [5] which is a rare carotenoid in higher plants. In this paper, the carotenoid composition of green and red cells and the effect of auxins and cytokinin on chromoplast formation are reported.

Experimental

Plant material

Ricinus cell cultures of strain A [4] were used which had been derived from the endosperm of the castor bean (*Ricinus communis*). The cells were cultivated in darkness or in fluorescent white light, respectively (Osram L 65 W/32; 5 Wm^{-2}) at 21 ° on a solid Gamborg B5 medium [6] supplemented with 2% sucrose and the auxins indoleacetic acid (0.5 mg/l), naphthaleneacetic acid (0.5 mg/l), 2,4-dichlorophenoxyacetic acid (2 mg/l) and 6-benzyl-

Table I. Composition of culture media (mg/l).

	A	B	C	D
6-Benzylaminopurine	0.2	0.2	0.2	2.5
2,4-Dichlorophenoxyacetic acid	2	–	–	–
Indoleacetic acid	0.5	–	0.5	4.0
Naphthaleneacetic acid	0.5	–	0.5	–
Inositol	100	100	100	100
Thiamine-HCl	10	10	10	–
Pyridoxine-HCl	1	1	1	0.5
Nicotinic acid	1	1	1	0.5
Glycine	–	–	–	2
Sucrose (g/l)	20	20	20	10
Caseine hydrolysate (g/l)	2	2	2	–

Each medium contains Gamborg B5 mineral salts.

aminopurine (0.2 mg/l) as sole cytokinin according to [7]. Modifications of this culture medium used in the differentiation experiments are summarized in Table I. Calli were subcultured every week.

Pigment analysis

Pigments were extracted from lyophilized cells with acetone. The chlorophyll concentration was determined from the absorbance at 652 nm according to [8]. Carotenoids were separated by TLC on silica gel plates with petroleum benzine (100–140°)/2-propanol (50:6; v/v) saturated with Na-methylate to obtain optimal separation of chlorophylls and carotenoids. The carotenoids had previously been identified by their absorbance spectra, mass spectra and by reference compounds [5]. Quantitative spectrophotometric analyses were performed according to [9] using $E^{1\%}_{1\,cm} = 2500$ at 486 nm for rhodoxanthin. For total carotenoids, an average coefficient of $E^{1\%}_{1\,cm} = 2500$ was used.

Electron microscopy

Cells were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4), postfixed in 1% OsO_4 in water for 2 h at 4° and dehydrated in an ethanol series. Additional staining was achieved with uranyl acetate followed by lead citrate. Cells were embedded in ERL (Serva). Sections were viewed in an EM 109 (Zeiss).

Results and Discussion

Effect of cytokinin and auxins

Cultures grown in the light and in the presence of cytokinin and auxins (medium A), were green and accumulated chlorophylls and carotenoids typical for chloroplasts; rhodoxanthin could be detected but was very low (Table II). The plastids contained stacked grana connected by stroma thylakoids. Generally, they had the same ultrastructural features as a typical higher plant chloroplast (Fig. 1). In this respect *Ricinus* cultures resemble many other green cultures, for instance, those of carrot [10], tobacco [11–14] and peanut [15], which require both cytokinin and auxins for the differentiation of chloroplasts.

In the dark, cultures were found to contain undeveloped plastids without internal membranes [16]. These cultures are characterized by a low content of carotenoids and by lack of chlorophylls and rhodoxanthin (Table II). This was found irrespectively of the presence of auxins (medium A or B).

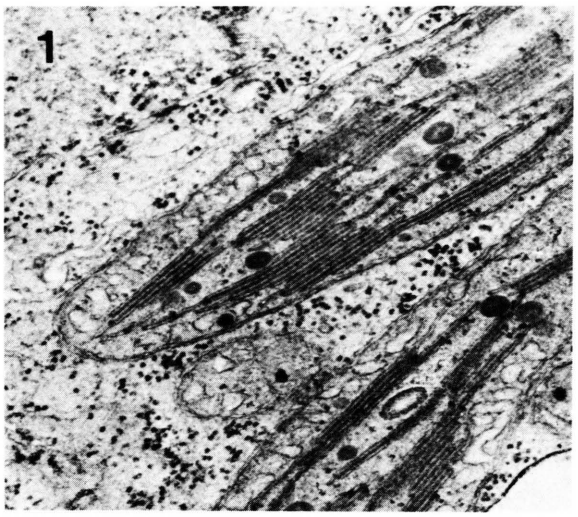
Effect of auxin reduction

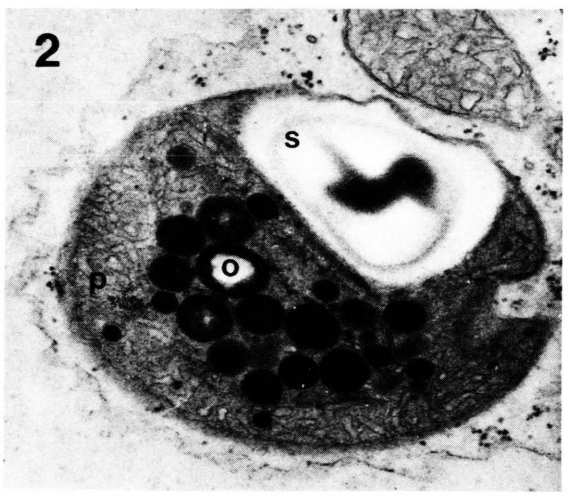
Pigment composition

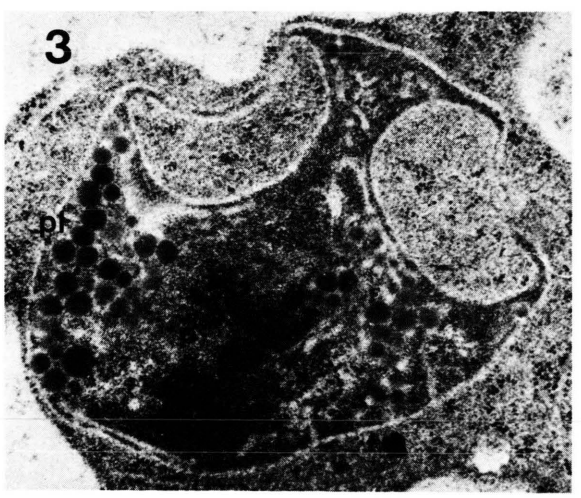
When cultures were transferred from the high-auxin medium A to media either without auxins (B) or with low auxins (C), or with high auxins in combination with enhanced cytokinin concentration (D), the cells turned to red. Since this effect depended on the ratio of cytokinin to auxins, it becomes evident that it is not the absolute auxin concentration but the combination of auxins with cytokinin which is responsible for this change. After a six-week culture on the low-auxin media

Table II. Effect of different concentrations of cytokinin and auxins on pigment composition (µg/g dry wt.). Calli were grown for 6 weeks on different media, as described in Table I. The data are mean values of 2–4 separate determinations.

	Dark	Light			
		Culture medium			
	A	A	C	D	B
Ratio cytokinin/auxins (w/w)	1:15	1:15	1:5	1:1.6	1:0
Total extract					
chlorophylls	0	329	1	30	4
carotenoids	30	171	177	190	192
Major carotenoids					
β-carotene	3	20	10	18	15
lutein	8	63	13	30	33
rhodoxanthin	0	2	95	91	99







B, C or D, respectively, the pigment composition of the red cells were analyzed. Chlorophyll levels were reduced to 9% on medium D and to trace amounts on B and C; carotenoids were slightly increased, for instance up to 112% on B and to 103% on C. In each culture the major carotenoid was rhodoxanthin which comprised approx. 70% of the total; lutein concentration was reduced to about 50% (Table II). Saponification of the total carotenoid extract provided no evidence for the presence of esterified xanthophylls. This is in contrast to senescing dark-grown *Ruta* cultures which contain up to 75% lutein esters [17]. The occurrence of oxygenated carotenoids, as reported from many other plant cell cultures (for review see [2]), seems to be a general phenomenon. On the other hand, as exception tobacco cell cultures accumulate the hydrocarbon lycopene [18].

Ultrastructure

Three weeks after transfer from medium A to B, chloroplasts were no longer found but there were plastids of irregular shape, some showing invaginations. Frequently, these plastids had starch grains and osmiophilic globules; the membranes were vesiculated (Fig. 2). This type of plastid has been reported represents the transition from a chloroplast to a chromoplast [1]. Additional to this type, amyloplasts were also found.

Six weeks after transfer to auxin-free medium, the plastids were devoid of internal membranes and contained considerable amounts of plastoglobuli. Many of these plastids were invaginated and had paracrystalline aggregates which were identified as phytoferritin [16] (Fig. 3). According to their structure and pigment composition, this type of plastids can be classified as globulous chromoplasts [19].

Fig. 1. Chloroplast of cells grown in the light in the presence of cytokinin and auxins (medium A). ×21,000.

Fig. 2. Plastid of cells grown for 3 weeks in the presence of cytokinin as sole hormone (medium B). ×16,000. s – starch, o – osmiophilic globules, p – phytoferritin.

Fig. 3. Chromoplast of cells grown for 6 weeks in the presence of cytokinin as sole hormone (medium B). ×28,000. pl – plastoglobules, p – phytoferritin.

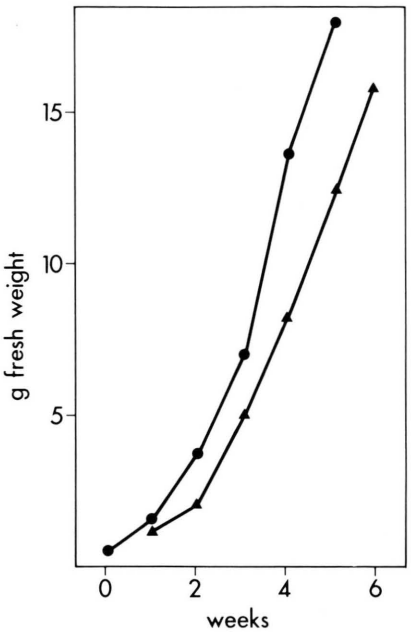


Fig. 4. Effect of the presence of auxins on growth rate of *Ricinus* callus cultures. Cultures were grown on auxin-containing medium A, (●) and on auxin-free medium B, (▲) respectively. Mean values of 4 independent cultures, variations were about 10%.

From this follows that in *Ricinus* cultures the transition of chloroplasts into chromoplasts is under hormonal control and can be induced by a reduction of the auxins. This was found to be valid also for the development of chromoplasts from relatively undeveloped plastids of dark-grown cultures (results not shown). In the intact plant it is supposed that the type of tissue (leaf, root, flower) determines what kind of plastid develops [3]. From our results, it is likely that hormones regulate plastid differentiation also in the intact plant.

Plastid interconversion

To get further insight into the transition process, pigment concentrations were followed during 5 weeks of culture without auxins; reversion experiments were also done.

First, it was found that omission of auxins had no significant effect on the growth of the cultures (Fig. 4); on the other hand, omission of cytokinin caused the decease of the cultures. This means that the observed effects are not due to degenerative processes as in senescing tissue like autumn leaves, ripening fruits or flowers [20], but reflect the activities of multiplying cells which produce continuously new plastids by division or differentiation of proplastids, respectively.

As shown in Fig. 5, the level of total carotenoids reached a minimum 1–2 weeks after transfer and by week 4 the initial level was reached. The concentration of lutein dropped down within two weeks from 75% to about 15%; concomitantly, there was a dramatic increase in the proportion of rhodoxanthin from 2% to 60%. β-Carotene decreased from 24% to 19%. In parallel to the decrease of lutein the chlorophylls disappeared (data not shown). Although the pathway of rhodoxanthin biosynthesis is still unknown, our data suggest that lutein might be

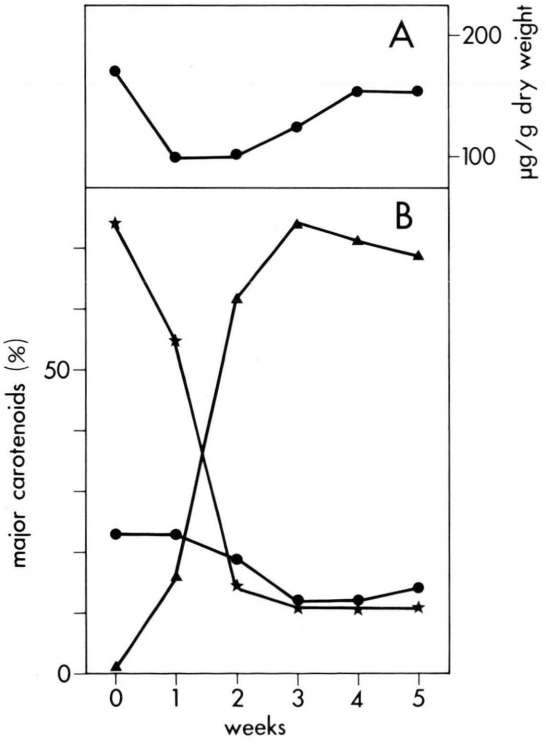


Fig. 5. Effect of auxin-deficiency on the amount of carotenoids of *Ricinus* callus cultures. Cultures were grown on auxin-containing medium A prior to transfer to auxin-free medium B at time 0. A. Sum of major carotenoids. B. Proportion of major carotenoids (▲, rhodoxanthin; ●, β-carotene; ★ lutein). For media composition see Table I. Mean values from 2 independent experiments.

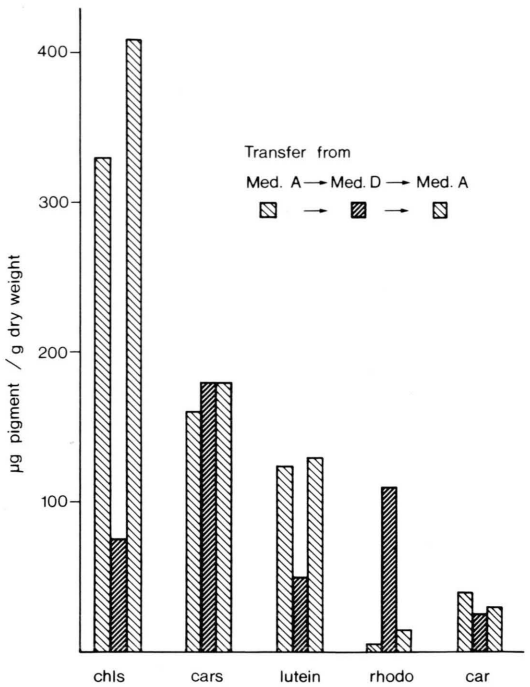


Fig. 6. Chromoplast reversal. Green calli were transferred from high-auxin medium A to low-auxin medium D. After five weeks on D one half was analyzed and one half was transferred back to A and analyzed another five weeks later. For media composition see Table I. Values are means from 2 independent experiments, chls – total chlorophylls, cars – total carotenoids, rhodo – rhodoxanthin, car – β-carotene.

involved. Provided this speculation is correct the hormones appear to induce or block steps subsequent to lutein leading to rhodoxanthin.

When green cultures were transferred to low-auxin medium for 5 weeks, then back to high-auxin medium and analyzed another 5 weeks later the pigment composition was found to be similar to that at the beginning of the treatment (Fig. 6). This shows clearly that the differentiation of the chromoplast can be reversed, and again it becomes clear that in *Ricinus* cell cultures the hormones determine which kind of plastid develops.

In conclusion, the present experiments show that cytokinin in concert with auxins promote the formation of chloroplasts.

Withdrawal of auxins exerts dramatic structural and metabolic changes resulting in the formation of chromoplasts. These changes include the production of a secondary carotenoid, the inhibition of chlorophyll synthesis, and the prevention of plastid membrane synthesis. The point at which primary regulation is imposed has not yet been revealed.

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