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Envelope Permeability to Possible Precursors of Carotenoid Biosynthesis during Chloroplast-Chromoplast Transformation[1]

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ABSTRACT

During the transformation of chloroplasts to chromoplasts in *Capsicum annuum* L., the permeability of the envelope membranes to possible precursors of carotenoid biosynthesis (acetate, mevalonate, citrate) was tested. The plastids were isolated by gel filtration, and the uptake of labeled compounds was measured by a filtering centrifugation technique, using silicone oil. The different ripening stages were characterized by the ratio of chlorophyll to carotenoid content. The chloroplast membranes were shown to be impermeable to all of the metabolites tested. During the transformation there was a sharp increase of membrane permeability. In the mature chromoplast, the permeability to mevalonate and acetate again decreased to about 20% of the maximum value and reached zero for citrate. The results give evidence that during the transformation of chloroplasts to chromoplasts, precursors for carotenoid biosynthesis are translocated from extraplastidic sites into the plastids, there being possibly incorporated into carotenoids.

A longstanding problem with respect to terpenoid biosynthesis is the question from where plastids get the precursors of carotenoid biosynthesis (acetate and mevalonate) and how this pathway is regulated.

For photosynthetically active tissue, Britton (4) suggested that there is a compartmentalization of terpenoid biosynthesis by a mechanism regulating the formation of different classes of terpenoids. This might involve enzyme segregation and specific membrane permeabilities to possible precursors.

As derived from studies with lettuce, oats, barley, and maize, there is an incorporation of [2-^{14}C]MVA[2] into the extraplastidic terpenoids such as squalene, sterols, and ubiquinone, whereas very little label is recovered in the chloroplast pigments, *e.g.* carotenoids, phytol, plastoquinones (8). The situation is reversed when the seedlings are grown in the presence of $^{14}CO_2$ Thus, the biosynthesis of a particular group of terpenoids in photosynthetically active tissue can occur only at one site; sterols, squalene, and ubiquinone are synthesized outside, carotenoids inside the plastid.

The question remains about the regulation of terpenoid biosynthesis in tissues, where CO_2 cannot be used for terpenoid biosynthesis within plastids. This problem arises when the development of chloroplasts or the transformation of chloroplasts to chromoplasts is studied, *i.e.* when the photosynthetic CO_2 fixation is not yet or no longer possible.

Wellburn and Hampp (17) have shown that the envelope membranes of early etiochloroplast stages of *Avena* are permeable to extraplastidic MVA and acetate, whereas there is an increasing impermeability to both metabolites during greening. Similar results were obtained using autoradiography at the level of electron microscopy (7).

Little work was done on chloroplast-chromoplast transformation, as well as on chromoplasts, with respect to the permeability of the envelope membranes to precursors of carotenoid biosynthesis. Only for chromoplasts the incorporation of [2-^{14}C]MVA into carotenes was shown with whole tomato fruits (1).

In this paper we present a contribution to the understanding of the compartmentalization of carotenoid biosynthesis during the transformation of chloroplasts to chromoplasts in *Capsicum annuum*. We investigated the possibility of the existence of a different membrane permeability to precursors of carotenoid biosynthesis during the process of transformation. Three possible precursors were applied: (*a*) mevalonate: shown to be a direct precursor for carotenoids (3, 9); (*b*) acetate: precursor for mevalonate via acetyl-CoA; (*c*) citrate: in this case we were interested in testing the possibility of citrate being translocated into the intraplastidic space. There it could be transformed to oxaloacetate and acetyl-CoA by a mechanism similar to that found in the cytoplasm:

$$\text{citrate} + \text{CoA} + \text{ATP} \rightleftharpoons \underset{\downarrow\;\downarrow\;\text{MVA}}{H_3C\text{-}\overset{\|}{\underset{O}{C}}\sim\text{SCoA}} + \text{oxaloacetate} + \text{ADP} + P_i$$

MATERIALS AND METHODS

Plant Materials. *C. annuum* L. (var. Szegediner, grüner, fleischiger) was grown in the greenhouse. Twelve hr before starting the experiment, fruits of different stages of ripening were harvested and stored at 4 C in the dark.

Plastid Isolation. About 40 g of chopped tissue were homogenized in 100 ml of chilled isolation medium (sorbitol, 350 mM; $MgCl_2$ 1 mM; EDTA, 1 mM; cysteine, 3 mM; BSA, Sigma Type V, 0.2% [w/v]; tris, 50 mM, adjusted to pH 7.3). The homogenate was filtered twice through four layers of nylon sheet (Nytal 25 T1, mesh 35 μm, Henry Simon, Stockport, U.K.), and centrifuged for 5 min at 1,000*g*. The pellet was resuspended in 4 ml of isolation medium and applied to a Sephadex G-50 column as described earlier (10). Only the first two fractions of the plastid eluate (8 ml) were taken from the column for each experiment. Contamination by mitochondria was routinely tested by Cyt *c* reductase assay (16) and was less than 5%. All procedures were carried out at temperatures ranging from 0 to 4 C.

Measurement of Penetration of Labeled Compounds Across the Plastid Envelopes. All incubations were run on ice, using isolation medium. To 1 ml of plastid suspension, corresponding to about 2 mg of protein, were added DL-[2-^{14}C]-labeled MVA, acetate or citrate (The Radiochemical Centre, Amersham, U.K.) to give a final concentration of 5 mM and a specific radioactivity of 0.6 mCi·mmol^{-1}. The amount of penetration of

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[2] Abbreviations: MVA: mevalonic acid; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A.

labeled compounds across the plastid envelopes in 1 min was measured by the silicone oil filtration method as described earlier (10). Uptake, by controls, of [^{3}H]H_2O and nonpermeating [U-^{14}C]sorbitol was measured in order to calculate plastid volumes and to correct for unspecific permeation into the inner membrane space, as well as for medium adhering to the outer surface of the organelles. Radioactivity was measured by liquid scintillation counting (ABAC SL 40, Intertechnique).

Extraction of Pigments. Three ml of plastid suspension were extracted first with ether, then with acetone until the respective extracts were colorless. Chl was measured according to Arnon (2). The combined pigment extracts were saponified with 6% KOH in methanol at room temperature for 2 hr. The chilled alkaline solution was extracted with ether. Finally, the ether extracts were washed alkali-free and dried over powdered anhydrous sodium sulfate.

Determination of Pigments. Approximate values for the relative amount of carotenoids of the different ripening stages were obtained by measuring the optical densities of the different extracts at their main absorption peak in ether. The calculation was carried out according to Jensen (14), using $E^{1\%}_{1\,cm}$ = 2,500 for green tissue, $E^{1\%}_{1\,cm}$ = 2,000 for the transition state, and $E^{1\%}_{1\,cm}$ = 2,000 for the red fruit. An additional determination for the red fruit was performed in hexane according to the method of Kirk and Juniper (13).

RESULTS

The possibilities to correct for the different plastid spaces (*i.e.* space between inner and outer envelope membranes, intraplastidic space) are relatively limited because of the different nature of the compounds used to estimate plastid spaces and uptake (sorbitol, organic acids), implying unequal binding properties to organelle membranes. Therefore, the levels of association of labeled metabolites with plastids are given in relation to the total volume of the plastids ([^{3}H]H_2O space, Table I), as well as to the sorbitol-impermeable space (space within the inner envelope membrane, Table II).

Calculated on the basis of the water-permeable space of the organelles, there is little label associated with chloroplasts and chromoplasts after an incubation time of 1 min at 0 C. The amount of label bound to mature chloroplasts and chromoplasts is very similar for MVA, acetate, and citrate.

In contrast, the different stages of chloroplast-chromoplast transformation (given as carotenoid to Chl ratio) show an enhanced binding of label to the water-permeable space of plastids. This is true for all metabolites tested, but most expressed for MVA. Stages of transformation, having a carotenoid to Chl ratio > 2.9 again show decreased levels of organelle-associated label.

The values of the uptake of ^{14}C-labeled MVA, acetate, and citrate into the sorbitol-impermeable space of plastids are given in Table II. They are corrected for amounts of metabolites adhering to the outer surface or taken up into the envelope space. Therefore, these rates of uptake should be a measure for the permeability properties of the inner envelope membrane, which is discussed to be transport-limiting (11). According to this, the inner envelope membrane of *Capsicum* chloroplasts is impermeable to MVA, acetate, and citrate under the conditions used. With an increasing carotenoid to Chl ratio, there is a pronounced increase of the intraplastidic concentration. The amount of uptake of MVA is highest for carotenoid to Chl ratios between 1 and 3, reaching levels similar to the medium concentration. An uptake of acetate is only measurable with chloroplast-chromoplast stages exceeding a carotenoid to Chl ratio of 1.66.

A low permeability for citrate occurs at late stages of chloroplast-chromoplast transformation (carotenoid to Chl ratio = 2.9). Chromoplasts again show a significantly reduced permeability of their inner envelope membranes, which is about 20% of the highest values measured for MVA and acetate during chloroplast-chromoplast transformation.

Table I. Plastid-associated levels of metabolites

These measurements were made after incubation of different chloroplast-chromoplast stages in 5mM solutions of ^{14}C-labeled mevalonate, acetate, and citrate. The values are calculated on a volume basis (tritiated water space of the organelles); ± = standard deviation (n=10). Measurements were carried out at 0 c.

Developmental stage (carotenoids/chlorophyll)	Plastid-associated label Mevalonate	Acetate	Citrate
	nmol·µl ^3H_2O-space^{-1}·min^{-1}		
0.02 (chloroplasts)	3.6 ± 0.4	2.3 ± 0.1	1.9 ± 0.2
0.50	4.0 ± 0.2	2.9 ± 0.2	2.3 ± 0.1
0.52	4.0 ± 0.1	3.0 ± 0.1	2.3 ± 0.1
1.02	4.7 ± 0.2	2.7 ± 0.1	2.4 ± 0.2
1.66	7.1 ± 0.5	4.4 ± 0.1	2.8 ± 0.2
2.92	6.0 ± 0.4	5.3 ± 0.2	3.9 ± 0.2
∞ (chromoplasts)	3.6 ± 0.1	2.9 ± 0.1	2.3 ± 0.1

Table II. Uptake of ^{14}C-labeled mevalonate, acetate, and citrate into the sorbitol-impermeable (intraplastidic) space of different chloroplast-chromoplast stages

External concentration of the respective metabolite: 5 mM; ± = standard deviation (n=10); - = no uptake at all. Measurements were carried out at 0 c.

Developmental stage (carotenoids/chlorophyll)	Rate of uptake Mevalonate	Acetate	Citrate
	nmol·µl intraplastidic space^{-1}·min^{-1}		
0.02 (chloroplasts)	-	-	-
0.50	1.3 ± 0.1	-	-
0.52	1.3 ± 0.1	-	-
1.02	2.0 ± 0.1	-	-
1.66	4.4 ± 0.3	1.7 ± 0.1	
2.92	3.3 ± 0.2	2.6 ± 0.1	1.2 ± 0.1
∞ (chromoplasts)	1.2 ± 0.1	0.5 ± 0.1	-

DISCUSSION

The results presented demonstrate a change of the permeability of the envelope membranes to MVA, acetate, and citrate during the transformation of chloroplasts to chromoplasts. The chloroplast membranes are impermeable to all of the precursors tested. During the plastid transformation there is a sharp increase of membrane permeability, which reaches a maximum and then falls to about 20% of the respective maximum value when the chromoplast stage is developed.

The finding that the envelope membranes of mature chloroplasts are a barrier for MVA transport confirms earlier results of Rogers *et al.* (15) and thus substantiates the suggestion that in photosynthetically active tissue the precursors of plastidic terpenoids are supplied via CO_2 fixation. The impermeability to acetate and citrate is a further point of evidence.

During the transformation of chloroplasts to chromoplasts the activity of photosynthetic CO_2 fixation decreases. Therefore, precursors of carotenoid biosynthesis, delivered by photosynthetic processes, become less available. Nevertheless, large amounts of carotenoids are synthesized when chromoplasts are formed. There should exist another pathway for carotenoid biosynthesis within plastids.

Two possibilities are to be considered: (*a*) degradation of lipids or other compounds stored inside the plastid; (*b*) import of precursors from extraplastidic sites. According to the results given in this paper, we assume that with the beginning of the transformation of chloroplasts to chromoplasts, MVA and acetate are translocated from extraplastidic sites into the intraplastidic space.

It is a well known proposal (12) that acetate, which is required for chloroplastic synthesis of fatty acids and terpenoids, originates from cytoplasmic sites. Our results indicate such an acetate import, but only for the stages of transformation of chloroplasts to chromoplasts.

With respect to the very limited envelope permeability to citrate during all stages of plastid transformation, this metabolite is suggested at least not to be a precursor imported from outside the plastid.

The assumption that with the beginning of transformation of chloroplasts to chromoplasts acetate and MVA are delivered from outside the plastids, is parallelled by investigations concerning the development of chloroplasts out of etioplasts. Wellburn and Hampp (17) showed that the envelope membranes of early etiochloroplasts are permeable to MVA. This was followed by a progressive impermeability to MVA and acetate during the later stages of greening.

It is of interest to compare our results with recent ones concerning the biosynthesis of MVA. Brooker and Russel (5, 6) demonstrated the reduction of HMG-CoA, forming MVA, with cell fractions of *Pisum* seedlings. Differential centrifugation studies showed that the microsomal fraction contained 80% of the total cellular activity of HMG-CoA reductase, whereas the mitochondrial and plastid fractions each contained about 10%. Of particular interest in this study is the more than 2-fold increase in microsomal HMG-CoA reductase activity in etiolated seedlings, compared to green ones. The authors suggested that this large increase may be related to the changes in growth and development, which are initiated when etiolated seedlings are exposed to light. In contrast, there was no comparable change in mitochondrial and plastidic HMG-CoA reductase associated with greening. This could implicate that during chloroplast development the observed changes in envelope permeability to MVA (17) are closely related to a possible import of MVA from an eventually elevated pool within the microsomal fraction.

If this suggestion is a feature common to transformational stages of plastids, similar changes in microsomal HMG-CoA reductase activity should occur during the chloroplast-chromoplast transformation. To confirm this, further work has to deal with the following questions. During the process of transformation of chloroplasts to chromoplasts is there (*a*) an increased activity of HMG-CoA reductase; (*b*) an elevated pool of MVA; (*c*) an incorporation of extraplastidic MVA into carotenoids inside the plastid?

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