BIOSYNTHESIS AND FUNCTION OF PLANT LIPIDS

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Chemical Induction of Carotenogenesis and Its Relationship to Chromoplast Differentiation in Cultured Plant Tissues

DONALD E. FOSKET, DAVID N. RADIN, and MARK GUILTINAN

In plants, carotenoid biosynthesis occurs in plastids. The nature of the carotenoids produced by the plant depends in part upon the differentiated characteristics of the plastids (10). Chloroplasts contain primarily small amounts of α- and β-carotene, and xanthophylls. In contrast, chromoplasts synthesize and store massive amounts of carotenoids, which frequently are different from those found in the chloroplasts or other plastids of the same plant (21). The differentiation of chromoplasts from chloroplasts represents a remarkable example of organelle transformation in which both the morphology and biochemistry of the plastid are changed radically in a short period of time. In some instances, this transformation is reversible, and the chromoplasts can again differentiate to become chloroplasts (22).

Over the course of the past decade, it has been demonstrated that carotenoid biosynthesis and accumulation can be induced in intact plants and plant organs by a class of synthetic compounds termed "bioregulators" by Hsu *et al.* (8). The bioregulators are substituted amines, such as 2-(4-chlorophenylthio-) triethylamine (CPTA) (16, 17). Bioregulators usually bring about a substantial increase in the total carotenoids of

treated plants. When the bioregulator CPTA is used, lycopene generally is the principal carotenoid formed (3, 8, 9).

Most of our information on the regulation of carotenoid formation in higher plants has come from an analysis of tomato fruit pigment mutants (10, 18). Mutations affecting 10 independent nuclear genes have been found in various tomato cvs which alter the fruit carotenoids (Table I). With the exception of the "ghost" and *v* mutations, all of these mutants affect only the kinds and amounts of carotenoids produced by the fruit chromoplasts. They do not affect chloroplast carotenoids.

These 10 genes tentatively have been assigned a regulatory or structural role in carotenogenesis, depending upon the kinds and amounts of carotenoids produced and upon the response of the mutant fruits to CPTA. Both *at* and *r* reduce the level of fruit carotenoids and neither produces lycopene, but this block can be overcome by spraying the mutant fruit with CPTA (10). Since all the biosynthetic functions seem to be intact, these mutants are thought to affect regulatory functions.

Because of the availability of these carotenogenesis mutants, we were particularly interested in developing an *in vitro* system for studying carotenogenesis in tomato. Our aim was to develop an experimental system for investigating the regulation of carotenogenesis and chromoplast differentiation at the molecular level. In this paper, we show that bioregulators can be used to study carotenogenesis and chromoplast differentiation in callus tissue and cell suspensions from many plants, including normal red-fruited and carotenoid mutant tomatoes.

MATERIALS AND METHODS

Callus and Cell Cultures. Callus tissues of *Rosa* sp. cv Paul's scarlet, and *Lycopersicon esculentum* cvs EP-7, LA-215, and 2-141 were cultured on agar-solidified media in the dark at 22°C or under cool-white fluorescent light with an 18-h photoperiod. The tomato tissues were cultured on modified Murashige-Skoog (MS) (13) medium as described previously (7, submitted), while the rose callus was cultured on Nesius' medium (15) with modifications (12). Suspension cultures of tomato were started by placing approximately 0.5 g of callus in 150 ml of liquid modified MS medium in a 500-ml Erlenmeyer flask. The cell suspension was filtered

Table I. Genes Affecting Carotenogenesis in Tomato[a]

Gene	Name	Characteristics
r	yellow fruit	No lycopene, 5% of the normal carotenoid level
at	apricot	No lycopene, 10% of the normal carotenoid level
v	yellow fruit	Regulates chloroplast and chromoplast carotenoid synthesis
gh	ghost	No carotenoids beyond phytoene. No utilization of phytoene in chloroplasts and chromoplasts to form colored carotenoids
og^c	crimson	Affects conversion of phytoene to colored carotenoids in chromoplast
t	tangerine	Conversion of *trans*-precursors of lycopene to the *cis* form, resulting in replacement of lycopene by prolycopene
vo	virescent orange	Conversion of zeta-carotene to neurosporene
B	beta-carotene	High levels of β-carotene. Mutant affects the cyclization of lycopene to produce β-carotene
mo$_B$		Modifies the expression of the *B* gene
Del	delta-carotene	Controls cyclization of lycopene to produce δ-carotene
hp	high pigment	Affects biogenesis of plastids

[a]Adapted from Kirk and Tilney-Bassett (10).

through a sterile stainless steel screen to remove large clumps. Thereafter, batch suspension cultures were started by pipetting 10 ml of a stationary-phase cell suspension into 140 ml of freshly prepared medium in a 500-ml Erlenmeyer flask. The suspension cultures were grown on a gyratory shaker which made 120 cycles/min at 22°C in the dark.

Carotenoid Extraction and Analysis. Carotenoids were extracted from the tissue by a modification of Davies' method (4). Tissues were weighed, frozen on dry ice, and stored at -20°C for later analysis. The frozen samples were thawed in acetone (15 ml/g fresh weight) and disrupted in the acetone with an Omni-Mix blender. The homogenate was filtered through a scintered glass filter. The acetone extract was collected and

saved while the residue was re-extracted with fresh acetone by homogenization for 1 min at top speed with the Omni-Mix blender. The tissue was extracted five times with acetone. The acetone extracts were combined, and the carotenoids were partitioned into an equal volume of light petroleum ether. The petroleum ether, containing the carotenoids, was washed repeatedly with water to remove the acetone, dehydrated by adding solid anhydrous sodium sulfate, and evaporated to dryness with a stream of N_2 gas. The carotenoids were dissolved in petroleum ether, and the volume was made up to 5 ml with petroleum ether. The total carotenoid content of the sample was determined spectrophotometrically as described by Davies (4). The Chl content of the samples was also determined spectrophotometrically as described by Strain and Svec (20).

Pigments were further analyzed by HPLC by a modification of published methods (6, 24). A Waters model 204 liquid chromatograph equipped with a Waters 10-μ C18 reverse-phase column was used for the separation of the tomato EP-7 carotenoids, while a Beckman model 334 liquid chromatograph with a 5-μ C18 reverse-phase column was used for the 2-141 and LA-215 tomato carotenoids. In either case, the instruments were operated in the isocratic mode with 100% acetonitrile as the mobile phase and the detector set to measure absorbance at 436 nm. The amount of β-carotene and lycopene in each sample was determined by measuring the areas under the peaks for each of these compounds and comparing these figures to standard curves constructed by analyzing known quantities of authentic lycopene and β-carotene.

Electron Microscopy. Tissues were minced in culture medium (minus growth substances) containing 1% glutaraldehyde at room temperature. The tissues were allowed to remain in this fixative for 2 h at room temperature, whereupon the fixative was replaced with 3% glutaraldehyde in tissue culture medium. After 4 h at room temperature in the 3% glutaraldehyde, the tissues were washed repeatedly with cold tissue culture medium and then placed in 0.05 M Pipes buffer, pH 6.8, at 4°C overnight. The tissues were postfixed for 1.5 h in 2% osmium tetroxide in 0.05 M Pipes buffer, pH 6.8, followed by three washes with 0.05 M Pipes buffer. The tissues were dehydrated in a graded ethanol series, followed by 50% ethanol in propylene oxide and finally in 100% propylene oxide. The

tissues were infiltrated and embedded in a graded series of Epon-Araldite in propylene oxide and polymerized at 60°C. Silver sections were cut with a Sorvall MT-2 microtome. The sections were poststained with uranyl acetate in methanol and then examined with a Zeiss 9S-2 electron microscope.

RESULTS

The Effect of CPTA on Callus Tissues. We have tested the effect of CPTA at concentrations ranging from 5 mg/L to 500 mg/L upon the growth and carotenoid content of callus tissues of carrot, rose, lemon, and tomato (submitted). In each case, the bioregulator was incorporated into the culture medium prior to inoculating the plates with the tissues. The callus was grown on the experimental medium for 21 d in the dark, after which the tissues were weighed and the carotenoids were extracted and determined. In every case, the tissues cultured on the medium containing bioregulator exhibited substantially higher levels of carotenoids.

The response of callus from the various species differed in detail. However, the general nature of the response is illustrated by the data presented in Table II for Paul's scarlet rose callus. At low concentration, CPTA had a relatively small effect on tissue growth, although high CPTA levels inhibited growth markedly. All levels of CPTA tested brought about a substantial increase in the carotenoid content of the tissue. At the optimun concentration for carotenoid formation, the tissues contained over 100 times the carotenoid content of the control tissue. Even at the lower CPTA concentration, which had only a small effect on growth, the carotenoid levels were elevated many-fold.

Induction of Carotenogenesis in Callus of a Normal Red-Fruited Tomato Cv. We tested the effect of CPTA on growth and carotenogenesis in callus tissue derived from the tomato cv EP-7. CPTA was incorporated into the medium at concentrations from 50 to 800 mg/L. Stationary-phase callus was transferred to the experimental media, and the tissues were cultured in the dark for 21 d. The fresh weight and carotenoid content of control and experimental tissues were determined and are shown in Figure 1. As was found with Paul's scarlet rose callus, higher levels of CPTA inhibited the growth of the callus somewhat, as indicated by the reduced final fresh

Table II. The Effect of CPTA on the Carotene Content of Paul's Scarlet Rose Callus

Stationary-phase Paul's scarlet rose callus was transferred to medium either lacking or containing CPTA at the indicated concentration. Twenty-one d later, fresh weights were determined. Carotenoids were extracted and determined as described in "Materials and Methods" (data taken from Radin, Guiltinan, and Fosket, submitted).

CPTA Concn (mg/L)	Final Fresh Weight of Tissue/Sample (g)	Carotenoid Content (µg/g fresh weight)	Increase in Carotenoid Content Due to CPTA (fold)
0	3.95	0.38	—
5	3.23	16.33	42.9
20	3.11	22.94	60.4
100	2.65	29.71	78.2
250	1.60	40.86	107.5
500	0.68	30.10	79.2

weights. However, all CPTA concentrations tested increased the carotenoid content of the tissues. At the optimum concentration for carotenoid formation (200 mg/L), the final tissue fresh weight was reduced by 52%. However, at a concentration of 100 mg/L (or 0.4 mM CPTA), there was no detectable effect on growth while the carotenoid content of the tissue was increased substantially.

The cells of the EP-7 tomato also may be grown as a suspension culture. The growth kinetics of the EP-7 cell suspension cultures are shown in Figure 2. When a stationary-phase culture was diluted 15-fold with fresh medium, the cultures exhibited a 2-d lag period, after which logarithmic growth was initiated. The log phase of growth persisted for at least 12 d.

Carotenoids were extracted from the cells at various intervals during the culture period. Regardless of the phase of the culture cycle, control cells exhibited the same basal level of total carotenoids. When CPTA was added to the culture medium at the start of the culture cycle,

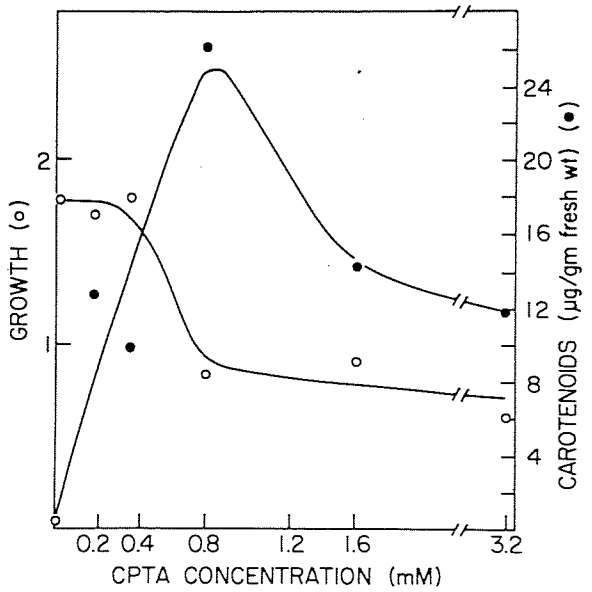

FIG. 1. The effect of CPTA on the growth and carotenoid content of callus derived from the red tomato cv EP-7. Callus tissue of tomato cv EP-7 was transferred to fresh medium which either lacked a bioregulator or contained CPTA at the indicated concentration. The cultures were grown for 21 d in the dark, after which the tissues were weighed and the carotenoids were extracted as described in "Materials and Methods." Each point represents an average of four determinations (data taken from Radin, Guiltinan, and Fosket, submitted).

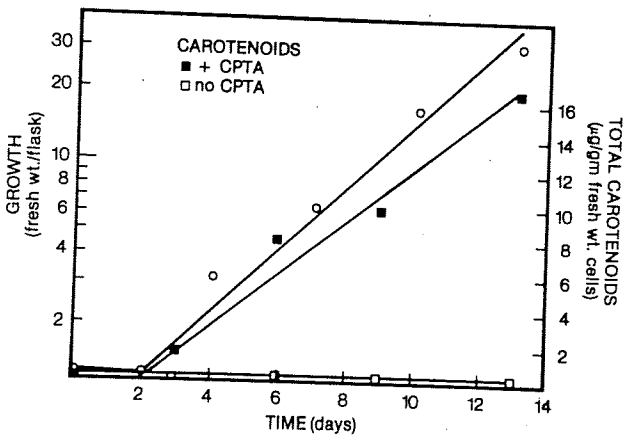

FIG. 2. Growth and carotenoid content of tomato cells of the red-fruited cv EP-7 in suspension culture. Stationary-phase tomato suspension cultures were diluted 15-fold with fresh medium in 500-ml Erlenmeyer flasks. The cell suspensions were cultured in the dark on a gyratory shaker completing 120 cycles/min. Some of the flasks contained 0.4 mM CPTA. Samples were taken at intervals for fresh weight (○) and carotenoid determination (□ or ■) (data taken from Fosket and Radin [7]).

the carotenoid content of the cells increased after a 2-d lag period. The increase was linear and continuous throughout the remainder of the growth cycle (7).

We fractionated the EP-7 cell carotenoids by means of HPLC (Fig. 3). The mobilities of peaks 1 and 2 were identical to those of authentic β-carotene and lycopene standards, respectively, when these were chromatographed under the same conditions. Control tomato cells contained mainly β-carotene and lycopene (Fig. 3B). Cells cultured in the presence of CPTA exhibited a progressive increase in total carotenoids, amounting to approximately a 65-fold increment at the end of the culture period; in addition, the composition of the carotenoids also changed. In the controls, the carotenoids were 75% lycopene and 15% β-carotene. As the carotenoid content of the CPTA-treated cells increased, the proportion of lycopene also increased until lycopene made up 93% to 94% of the samples and β-carotene represented only 5% (Fig. 3, C and D) (7).

When CPTA was added to log-phase cultures, carotenoid biosynthesis was initiated after a 3-h lag period. By the 6th h after adding CPTA to the cultures, the carotenoid content of the EP-7 cells had more than doubled. Carotenoids accumulated at the rate of 0.6 µg of carotenoids/h/g fresh weight.

If cycloheximide was added to the log-phase cultures, along with the CPTA, much of the CPTA-induced increase in carotenoid content was prevented. The magnitude of the effect of cycloheximide in blocking the CPTA-induced carotenoid increase was similar to the effectiveness of the same concentration of cycloheximide in blocking ^{3}H-leucine incorporation into protein. However, the cycloheximide had no effect on the carotenoid content of the control cells (Table III).

Induction of Carotenogenesis in Callus Derived from Mutant Tomatoes. We have initiated callus tissue from two tomato cvs homozygous for genes involved in carotenogenesis. These are the cvs 2-141 and LA-215, known respectively as "yellow fruit" and "apricot fruit." These tissues were transferred to fresh medium either containing or lacking 0.4 mM CPTA and cultured for 21 d in the dark. The tissues were weighed and pigments extracted, and they were analyzed by HPLC.

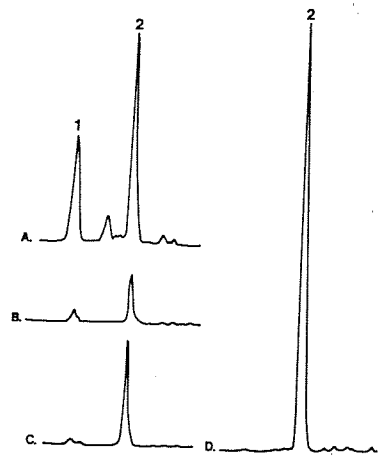

FIG. 3. HPLC analysis of carotenoids extracted from tomato fruit or cultured cells. Carotenoids were isolated from immature tomato fruit chromoplasts (A) and from cultured tomato cells of the EP-7 cv. The carotenoids were separated by HPLC as described in "Materials and Methods." The sequence of chromatographic elution was from right to left. Peaks 1 and 2 were shown to have the same mobilities and spectral properties as β-carotene and lycopene, respectively. A, Tomato fruit carotenoids; B, Carotenoids of untreated cultured tomato cells; C and D, Carotenoids from tomato cells after 3 (C) and 9 (D) d in medium containing 0.4 mM CPTA (data taken from Fosket and Radin [7]).

Table III. The Effect of Cycloheximide on CPTA-Induced Carotenogenesis

A stationary-phase tomato suspension culture was diluted 15-fold with fresh medium and grown on a shaker in the dark. Six d later, the culture was aliquoted, and the aliquots were treated as indicated below. Six h after the start of the treatments, the cells were harvested, weighed, and frozen in liquid nitrogen. Carotenoids were extracted and estimated as described in "Materials and Methods" (from Fosket and Radin [7]).

	Total Carotenoids (μg/g fresh weight)	% Increase	Lycopene (μg/g fresh weight)
Control, no CPTA	0.148	—	0.023
Control + 10 μM Cyc	0.152	2.7	0.029
Control + 100 μM Cyc	0.153	3.3	0.025
+CPTA (0.4 mM)	0.356	137.0	0.188
+CPTA + 10 μM Cyc	0.242	61.0	0.113
+CPTA + 100 μM Cyc	0.166	11.0	0.075

As shown by the data in Table III, we could not detect any carotenoids in control callus of the yellow fruit mutant by either spectrophotometry or by HPLC. When treated with CPTA, the yellow fruit callus did not change color and become either pink or red as did callus of other CPTA-treated tomato cvs. However, when 37 g of CPTA-treated callus were extracted as described, and the extracted pigments were concentrated into 5 ml of petroleum ether, we were able to detect a small amount of lycopene.

In contrast, callus tissue of the apricot fruit tomato mutant contained substantial amounts of carotenoids when cultured in the dark. Virtually all of these carotenoids were in the form of β-carotene. We were unable to detect any lycopene in these tissues. After 21 d in the dark on medium containing CPTA, the levels of β-carotene in the tissues were reduced by over 50%, and the callus contained substantial quantities of lycopene. The CPTA-induced stimulation of total carotenoids was small compared to that observed in callus derived from the red-fruited tomato. Where 0.4 mM CPTA brought about over a 60-fold increase in the total carotenoids of the EP-7 cv, the same concentration of CPTA elicited only

a 0.78-fold increase in callus of the apricot fruit mutant when cultured under identical conditions (Table IV).

Table IV. Pigment Content of Callus Derived from Mutant Tomatoes as Effected by CPTA

Callus tissues of the tomato carotenoid mutants 2-141 and LA-215 were transferred to fresh medium which either contained or lacked 0.4 mM CPTA. The callus cultured in the light was exposed to a daily 16-h photoperiod, during both the experiment and the previous subculturing period. The experimental callus cultured in darkness had been grown in darkness for many subculturing periods prior to this experiment. After 21 d, the callus was weighed, the pigments were extracted, and the content of lycopene and β-carotene was determined as described in "Materials and Methods."

Mutant[a]	Cultural Condition	µg Pigment/g Fresh Weight		
		Lycopene	β-carotene	Chl
2-141	Dark no CPTA	n.d.[b]	n.d.	n.d.
2-141	Dark + CPTA	0.169	n.d.	n.d.
LA-215	Dark no CPTA	n.d.	18.5	n.d.
LA-215	Dark + CPTA	24.65	8.05	n.d.
LA-215	Light no CPTA	n.d.	41.9	46.12
LA-215	Light + CPTA	17.18	3.22	n.d.

[a]Mutant 2-141 = r = yellow fruit; Mutant LA-215 = at = apricot fruit.
[b]n.d. = not detected.

Other differences in the behavior of the two mutant calli were noted, the most striking of which was their response to light. Callus of the yellow fruit mutant neither turned green nor produced additional carotenoids when cultured in the light. In contrast, callus of the apricot fruit mutant not only became green but also produced substantial quantities of xanthophylls and high levels of β-carotene when cultured in the light (Table IV). When the light-grown apricot fruit mutant callus was transferred to fresh medium containing 0.4 mM CPTA and the tissue was cultured in the light, there was a substantial reduction in the total carotenoids provided by the tissues. The CPTA treatment reduced the Chl and xanthophyll content of the tissues to undetectable levels and reduced the β-carotene levels by over 90%. At the same time, the callus was bright red and contained a substantial level of lycopene (see Fig. 4 and Table IV).

The Ultrastructure of CPTA-Treated Mutant Tomato Callus. We examined the ultrastructure of plastids produced by the apricot mutant callus when it was cultured in the light. As shown by the electron micrographs of Figure 5, the plastids are large and exhibit a fairly extensive development of thylakoids. In some places, the thylakoids are stacked into grana, but for the most part, the thylakoid membranes are exposed to the stroma rather than appressed. The stroma is fairly electron-dense. In some cases, the plastids contain large starch grains as well as thylakoids, while in other cases dense plastoglobuli are evident.

When callus of the apricot mutant was cultured in the light on medium containing CPTA, plastid morphology was altered dramatically (Fig. 6). The plastids are not only very much smaller, but there has been a substantial reduction in the number of thylakoids, and the stroma is more electron-dense. The most striking feature of the light-grown CPTA-treated plastids is the presence of large lycopene crystals. The number of crystals is fairly small, from 1 to 3, and they tend to run the length of the plastid.

DISCUSSION

Yokoyama and his co-workers have suggested that bioregulators such as CPTA have a dual effect on higher plants (8). The primary effect is

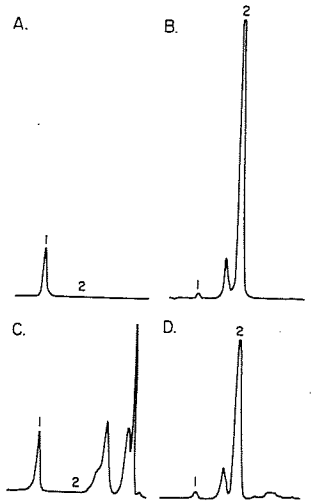

FIG. 4. HPLC analysis of carotenoids extracted from callus derived from the tomato mutant LA-215 (apricot fruit). Callus tissues which had been cultured in either continuous darkness or under a 16-h photoperiod were subcultured to fresh medium which either lacked a bioregulator or contained 0.4 mM CPTA. Plates inoculated with light-grown callus were returned to the 16-h photoperiod while plates inoculated with dark-grown callus were cultured in the dark. Twenty-one d later, the callus was weighed, pigments were extracted, and the extract was fractionated by HPLC as described in "Materials and Methods." The elution order was from right to left. Peak 1, ß-carotene; peak 2, lycopene. A, Dark-grown callus, no CPTA; B, Dark-grown callus, plus CPTA; C, Light-grown callus, no CPTA; D, Light-grown callus, plus CPTA.

FIG. 5. Ultrastructure of chloroplasts from light-grown callus of tomato cv LA-215 (apricot fruit). Seven d after transfer, callus tissue was prepared for electron microscopy. These micrographs illustrate the structure of chloroplasts containing starch grains (A, X 2,000) and chloroplasts without evident starch grains (B, X 20,000). cw = cell wall, s = starch grain, v = vacuole.

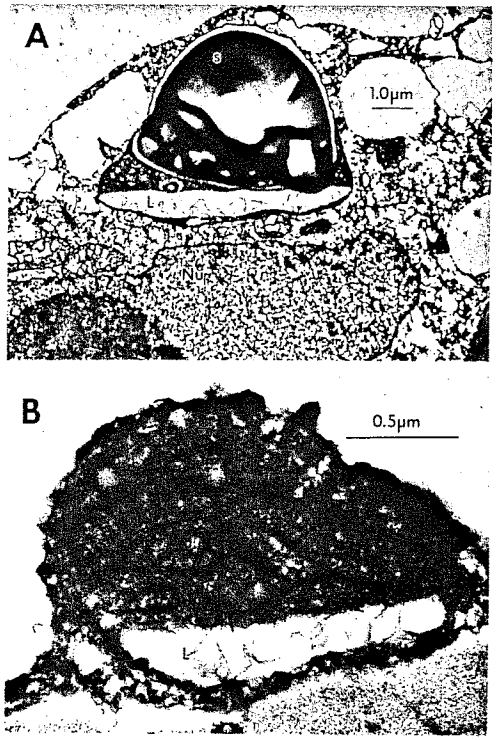

FIG. 6. Ultrastructure of chromoplasts from light-grown, CPTA-treated callus of tomato cv LA-215 (apricot fruit). Seven d after transfer to fresh medium containing 0.4 mM CPTA, samples were prepared for electron microscopy. These micrographs illustrate the morphology of plastids containing lycopene crystals (L), both in starch-containing plastids (A, X 9,100) and in plastids lacking evident starch grains (B, X 49,000). S, starch grain; Nu, nucleus.

proposed to be on gene expression. CPTA is postulated to initiate or enhance the expression of genes coding for enzymes of carotenoid biosynthesis. The fact that CPTA-treated plants accumulate lycopene is thought to be due to a secondary effect, namely, the inhibition of the cyclase enzymes that convert lycopene to β-carotene and the other cyclic carotenoids.

The evidence that bioregulators act at the level of gene expression is mostly indirect. Hsu et al. (8) demonstrated that the bioregulator-induction of carotenoid formation in the fungus Blakeslea could be blocked by the inhibitor of protein synthesis, cycloheximide. In the present study, we show that cycloheximide also blocked CPTA-induced carotenoid formation in higher plant cells. In addition, cycloheximide's capability to block the bioregulator action was proportional to its effectiveness in inhibiting ^{3}H-leucine incorporation into protein in the same system. Unfortunately, the interpretation of these results is complicated by the fact that cycloheximide has been found to have side effects on plant cells which are unrelated to the drug's ability to block protein synthesis (2, 5, 11). However, the fact that cycloheximide did not affect the carotenoid content of the control cells, and that most of its reported side effects appear only after treating at higher concentrations and over longer time periods than we have used, suggests that our interpretations of these results probably are valid.

Working with the fungus Phycomyces, Murillo (14) also demonstrated that inhibitors of protein synthesis blocked CPTA-induced carotenoid formation. However, he proposed that the bioregulator has only an indirect effect on gene expression. He suggested that if the carotenoid biosynthetic pathway is under negative control from a cyclic carotenoid end product, CPTA could stimulate total carotenoid production with the accumulation of lycopene by preventing the formation of the cyclic carotenoid end products, principally β-carotene. Regardless of the possible validity of this hypothesis for Phycomyces, it is an unlikely explanation for the CPTA-induction of carotenogenesis in EP-7 tomato callus, where CPTA treatment was found to enhance the levels of both β-carotene and lycopene. Bucholtz et al. (1) also argue against this mechanism. They found that CPTA was a weak inhibitor of total carotenoid synthesis in a cell-free

tomato fruit plastid preparation that incorporated labeled precursors into lycopene and β-carotene. Furthermore, they demonstrated that CPTA did not inhibit the activity of the cyclase enzymes in this cell-free preparation.

For these reasons, the most plausible explanation for CPTA-enhanced carotenogenesis in tomato callus is that the bioregulator acts at the level of the regulation of gene expression. While other hypotheses can readily be constructed for the action of CPTA, this hypothesis is both simple and supported by the available evidence. Kirk (10) has offered the most attractive hypothesis to date for a mechanism by which CPTA could stimulate the expression of genes coding for proteins in the carotenogenic pathway. He noted that lycopene was not formed during fruit ripening in the tomato mutants apricot or yellow fruit. However, CPTA treatment would elicit lycopene formation in the fruit of both of these tomatoes. He proposed that carotenogenesis was initiated during fruit ripening when a natural inducer combined with a regulatory protein. The regulatory protein-inducer complex would then enter the nucleus where it would combine with the chromatin containing genes for carotenoid biosynthesis, thus allowing it to be transcribed. The two mutant tomatoes, apricot and yellow fruit, were seen to have a defective regulatory protein which either would not bind the natural inducer or would bind it poorly. CPTA was proposed to have a much greater affinity for the regulatory protein than the natural inducer so that it could bind to the mutant regulatory protein where the natural regulator could not. As a result, CPTA not only is a more powerful inducer of carotenogenesis in normal plants but also can induce carotenogenesis in the regulatory mutants apricot and yellow fruit.

Our results are consistent with this hypothesis. Certainly we have demonstrated that CPTA was able to bring about lycopene synthesis in callus derived from both tomato mutants. Neither callus tissue contained detectable levels of lycopene in the absence of the bioregulator, in contrast to the callus derived from the normal red-fruited cv EP-7. Although callus of the yellow fruit mutant produced relatively little lycopene in response to CPTA, there is no doubt that this tissue did respond to the bioregulators. Callus of the apricot fruit mutant gave a more dramatic response. The lycopene levels in the CPTA-treated callus were

among the highest we have observed in any of the cultured cells we have used. However, lycopene was not detectable in the apricot fruit mutant callus when cultured on medium lacking a bioregulator.

When discussing the regulation of carotenogenesis, it is important to keep in mind that there is evidence for two distinct carotenogenic systems in higher plants. One is associated with chloroplasts and regulates the formation of β-carotene and other carotenoids that function in photosynthesis. The second system is associated with chromoplast differentiation. The evidence that these two pathways are regulated separately comes from both physiological and genetic studies. High temperatures, which have been shown to inhibit lycopene formation in developing tomato chromoplasts, have no effect on β-carotene synthesis by chloroplasts (23). As noted above, all tomato fruit carotenoid mutants (except gh and v) have no effect on β-carotene levels in leaves. The mutants gh and v operate earlier in the pathway, at or before phytoene synthesis, and thus affect both carotenoid-forming systems. Apparently, the genes or regulatory mechanism for carotenoids are common up to the point of phytoene synthesis. After phytoene synthesis, either the genes for the carotenogenic enzymes or the regulatory mechanism controlling the expression of these genes differs in the two carotenoid pathways.

The effects of the bioregulators usually include the elevation of total carotenoid levels. This is not invariably the case, however. Simpson and Lee (19) reported that the CPTA treatment of some immature Capsicum fruits actually reduced the total fruit carotenoid levels. In those cases, the lycopene content of the fruit tissue was enhanced, and the bioregulator brought about structural changes in the fruit chloroplasts characteristic of chromoplast differentiation. These effects may be similar to those we have observed in light-grown callus of the apricot fruit mutant. Although CPTA treatment initiated the synthesis and accumulation of substantial quantities of lycopene, the light-grown, CPTA-treated callus actually contained less total carotenoids than the light-grown, untreated control. This is because CPTA treatment brought about a dramatic reduction in the β-carotene content of the tissues and virtually eliminated the xanthophylls. The latter two types of carotenoids are characteristic of chloroplasts. Thus, while CPTA induced the formation of

chromoplast carotenoids, it inhibited the formation of chloroplastic carotenoids.

It is important to point out that CPTA treatment of the green, light-grown apricot fruit mutant callus also brought about structural changes in the plastids, which suggests that it induced chromoplast differentiation from the chloroplasts. Along with the apparent differentiation of the chloroplasts into chromoplasts, we observed the loss of structural features characteristic of the chloroplasts, such as the thylakoid membranes. The differentiating chromoplasts stopped producing both the chloroplast-specific carotenoids and Chl as well. These results suggest that the bioregulators activate the entire chromoplast differentiation pathway, of which carotenoid biosynthesis is only one part.

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