# Chromoplast-Specific Proteins in *Capsicum annuum*[1]

NOURÉDDINE HADJEB, IOANNIS GOUNARIS[2], AND CARL A. PRICE*
*Waksman Institute, Rutgers University, Piscataway, New Jersey 08855-0759*

## ABSTRACT

Chromoplasts are a common differentiation state of plastids in which the photosynthetic apparatus is absent and carotenoids accumulate to high levels. As a first step toward the isolation of chromoplast-specific genes, we have examined plastids of the bell pepper, *Capsicum annuum* L., for the presence of chromoplast-specific proteins. Intact chromoplasts were isolated from mature fruits of *C. annuum* var Emerald Giant, Golden Cal Wonder, and DNAP VS-12 by differential centrifugation followed by isopycnic sedimentation in gradients of silica sols. The plastids were then fractionated into soluble and membrane components and the proteins analyzed by one- and two-dimensional gel electrophoresis using isoelectric focusing, sodium dodecyl sulfate, and sodium dodecyl sulfate-urea gels. Two polypeptides with $M_r$ of 35,000 and 58,000 accumulate to high levels in membrane fractions of chromoplasts of var Emerald Giant. These polypeptides are either not detectable or barely detectable in chloroplasts from immature fruits. Both polypeptides have been purified to near homogeneity. Yellow chromoplasts from var Golden Cal Wonder and red chromoplasts from var DNAP VS-12 contained the 35-kilodalton polypeptide, but not the 58-kilodalton species.

In fruits of most red varieties of the bell pepper, *Capsicum annuum* L., chloroplasts differentiate into chromoplasts. During this process, thylakoids are sharply diminished and the grana are lost (8, 22); Chls disappear and carotenoids accumulate to high levels (2, 3, 6).

The color of mature fruits of *C. annuum* is determined by four nuclear genes (12, 13). Since the photosynthetic machinery disappears and new structures characteristic of chromoplasts are formed (15, 24), one would expect additional genes might be involved in chromoplast development. Although genes coding for steps in carotenoid biosynthesis have been isolated from photosynthetic bacteria (10), no genes controlling chromoplast development in higher plants have been isolated.

In previous experiments we detected abundant transcripts in chromoplasts, but the transcripts were from the same regions of plastid DNA as occur in chloroplasts of immature fruits (11); *i.e.* none of the transcripts were specific to the chromoplast itself. Our present strategy is to isolate chromoplast-specific proteins and, through these, to isolate chromoplast-specific genes of the
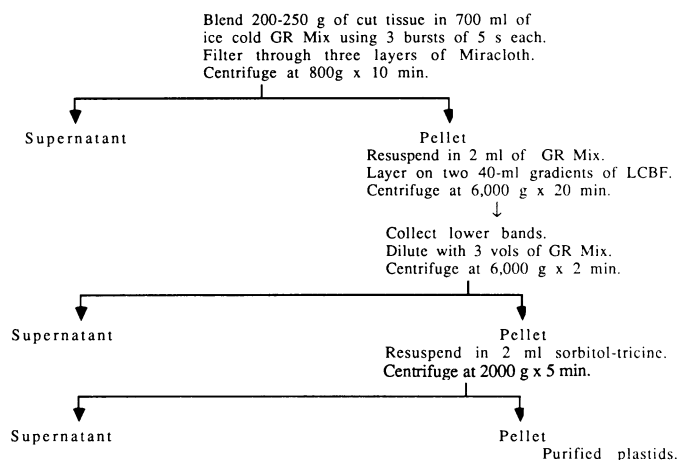
nucleus. Toward this end, we have compared the proteins of chromoplasts with those of chloroplasts by SDS-gel electrophoresis and isoelectric focusing.

We report here the presence of several polypeptides that accumulate to high levels in chromoplasts but are either not detectable or barely detectable in chloroplasts.

## MATERIALS AND METHODS

**Plant Material.** Green and red fruits of *Capsicum annuum* L., var Emerald Giant, Golden Cal Wonder, and DNAP VS-12, were grown in green house or in the field by Dr. Robert A. Morrison of DNA Plant Technology Corporation and were used within 3 to 5 d of harvest. The fruits were kept in the dark at 4°C to deplete starch grains.

**Isolation of Plastids.** Chloroplasts from green fruits were isolated according to Price *et al.* (20). Chromoplasts were isolated by combining features of the chloroplast procedure with the method of Camara *et al.* (4). Non-carpal tissues were removed from the fruits. The carpal tissue was cut into small pieces of about 1 cm², and 200 to 250 g were blended with 700 mL GR mix[3] (9) (1 mM $NaP_2O_7$, 50 mM Hepes, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM isoascorbate, pH 6.8). The clarified brei was centrifuged, the resulting pellet of crude plastids was suspended in GR mix, and the plastids were purified by isopycnic sedimentation in gradients of LCBF (Table I). The composition of LCBF is 100 mL Ludox AM (E. I. DuPont), 3 g polyethylene glycol (Carbowax 8000, Union Carbide), 1 g of bovine serum albumin (Sigma), and 1 g Ficoll (Pharmacia Fine Chemicals). Percoll may be substituted for Ludox AM with appropriate adjustments for the lower density of Percoll (20). The protocol is shown in Figure 1.

**Protein Extraction from Intact Plastids.** The purified plastid pellet prepared as described above was resuspended in 1 mL of deionized water and left at room temperature for 15 min and centrifuged at 6000g for 5 min. The pellet was resuspended in water and washed twice. The supernatants were combined as the soluble fraction. Samples were made 90% v/v in acetone (21), mixed at room temperature, and incubated on ice in the dark for 1 h. The suspension was then centrifuged at 5,000g for 15 min at 0°, and the pellet was washed twice with 10 mL acetone, centrifuged, dried under a stream of $N_2$, and stored at −20°C.

**Electrophoresis.** Electrophoretic procedures were generally those of Chua (5). For one-dimensional PAGE, the pellet was resuspended in 0.1 M $Na_2CO_3$-0.1 M DTT; SDS sample buffer (5% [w/v] SDS, 30% [w/v] sucrose, 0.1% [w/v] bromophenol blue) was added to each sample; and samples were boiled for 2 min prior to electrophoresis (14, 18, 21). One-dimensional electrophoresis was performed on 9 to 15% (w/v) linear gradients (17) or on gels of 5 M urea (acrylamide:bisacrylamide = 50:8) as described by Westhoff *et al.* (23). For two-dimensional PAGE, the pellet was solubilized in SDS as described (21). The first

[2] Present address: USDA, ARS, U.S. Regional Pasture Research Laboratory, University Park, PA 16802.

[3] Abbreviations: GR mix, grind-resuspension mix; LCBF, Ludox-Carbowax-bovine serum albumin-Ficoll.

Table I. *Constituents of Silica-Sol Gradients for the Isolation of Plastids from C. annuum*

| Ingredient | Starting Solution | | Limiting Solution | |
|---|---|---|---|---|
| | [Final] | Amount | [Final] | Amount |
| 5 × GR Mix | | 8 mL | | 8 mL |
| Glutathione | 5 mM | 7 mg | 5 mM | 7 mg |
| LCBF | | | | |
| Chloroplasts | 10% v/v *or* | 4 mL *or* | 79% v/v *or* | 31.6 mL *or* |
| Chromoplasts | 5% v/v | 2 mL | 40% v/v | 16 mL |
| Deionized water | to 40 mL | to 40 mL | to 40 mL | to 40 mL |

Blend 200-250 g of cut tissue in 700 ml of
ice cold GR Mix using 3 bursts of 5 s each.
Filter through three layers of Miracloth.
Centrifuge at 800g x 10 min.

Supernatant
Pellet
Resuspend in 2 ml of GR Mix.
Layer on two 40-ml gradients of LCBF.
Centrifuge at 6,000 g x 20 min.

Collect lower bands.
Dilute with 3 vols of GR Mix.
Centrifuge at 6,000 g x 2 min.

Supernatant
Pellet
Resuspend in 2 ml sorbitol-tricine.
Centrifuge at 2000 g x 5 min.

Supernatant
Pellet
Purified plastids.

FIG. 1. Method of isolation of plastids from fruits of *C. annuum*. The working temperature is 0 to 4°C throughout.

dimension of two-dimensional gel electrophoresis, isoelectric focusing, was performed essentially as described by Roscoe and Ellis (21), except that the tubes were 3 mm i.d. × 11 cm and contained 2% (w/v) Ampholines (LKB) consisting of 1.6% (w/v) pH range 3.5 to 10 and 0.4% (w/v) pH range 5.0 to 8.0. The second dimension was run on 9 to 15% (w/v) linear gradients in 2-mm SDS-polyacrylamide slabs.

**Protein Elution from Polyacrylamide Gels.** Protein bands of interest were eluted by the method of Russell Durbin (personal communication) as follows. Bands were cut out and washed with distilled water with two changes over 30 min. Proteins were extracted with four volumes of a mixture of 0.1 M Tris-HCl (pH 8.4), 1 mM Na₂EDTA, 0.2% SDS, and 0.1% 2-mercaptoethanol at 37°C with gentle agitation for 24 h. The extracts were filtered through a 0.4-μm Millipore filter, after which the samples were treated with 0.2 mL of 0.1 M quinine bisulfate per mL, gently rotated without delay, and left standing for 10 min at room temperature. The preparations were centrifuged at approximately 10,000 rpm in the microcentrifuge, the supernatant was discarded, 1 ml of acetone was added to the pellet, and the mixture was left standing for 10 min. The suspensions were vortexed until all of the pigment was solubilized. The pellets were extracted three more times.

## RESULTS

**Isolation of Plastids.** Pepper fruits were disrupted in an isoosmotic buffer and the plastids were isolated by a combination of differential centrifugation and isopycnic sedimentation in gradients of silica sol as described in Figure 1. Gradient tubes contained two prominent pigmented bands (Fig. 2). The lower bands from green and red fruits contained intact chloroplasts and chromoplasts, respectively, as shown by refringence in phase-contrast or Nomarski-contrast microscopy. Whereas chloroplasts appeared quite stable under the microscope, chromoplasts from
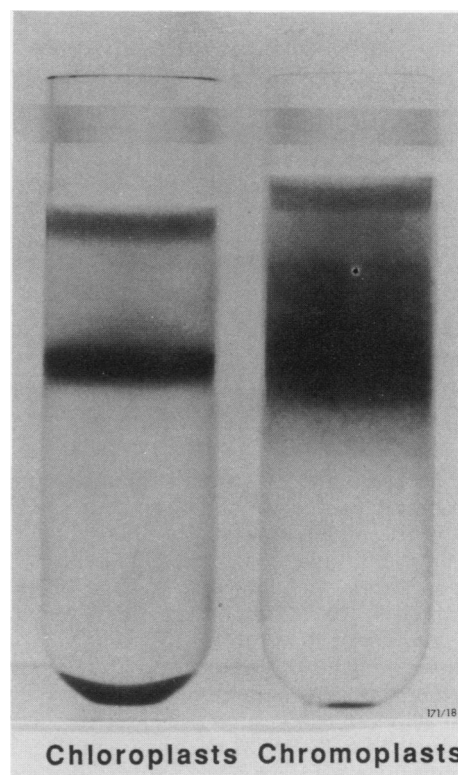


**Chloroplasts  Chromoplasts**

FIG. 2. Isopycnic sedimentation of plastids in silica-sol gradients. Crude plastids were purified by differential centrifugation and sedimented to equilibrium on gradients of Ludox AM. The upper bands in each case contain plastid membranes and the lower bands mostly intact plastids. Chloroplasts are from green fruits; chromoplasts are from red fruits. Note: the gradient concentrations are different to allow for the lower densities of chromoplasts compared to chloroplasts.

red fruits were somewhat fragile, even when maintained in an isotonic buffer; chromoplasts from yellow fruits were considerably more fragile.

**Analysis of Plastid Proteins.** Isolated plastids of var. Emerald Giant were resolved into membrane and soluble fractions by sedimentation. Extracts of total plastids, membranes, and soluble proteins were analyzed by electrophoresis on SDS-polyacrylamide gradient gels (Fig. 3). The total protein fraction of chloroplasts displays an array of polypeptides typical of chloroplasts generally. The membrane fraction contains prominent components characteristic of the Chl *a/b*-binding proteins. The yield of the soluble proteins was low. The polypeptide pattern of total chromoplast extracts is distinct from those of the total chloroplasts: two major polypeptides of $M_r$ 35,000 and 58,000 are predominant. Both species are enriched in the membrane fractions but also occur in the soluble fractions. Chloroplasts in contrast contain little or none of the 35- and 58-kD polypeptides.

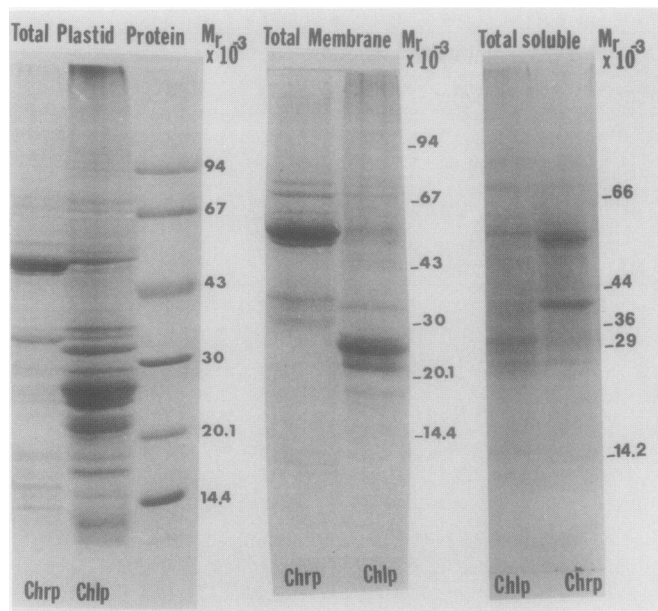In order to compare further the polypeptide patterns of pepper

FIG. 3. SDS-PAGE of chromoplast and chloroplast proteins from *C. annuum.* Proteins were subjected to electrophoresis on a 9 to 15% polyacrylamide gradient gel. Left, total plastid proteins and molecular weight markers; center, membrane proteins; right, soluble proteins; Chlp, chloroplast; Chrp, chromoplasts.

plastids, we carried out electrophoresis in urea, which differentially affects mobilities of hydrophilic and hydrophobic proteins. The chromoplast components whose mobilities correspond to $M_r$ values of 35,000 and 58,000 in SDS-polyacrylamide gels migrated in urea as polypeptides of 28,000 and 46,000 to 50,000, respectively (Fig. 4). No corresponding bands were observed in the chloroplast preparation.

Isoelectric focusing affords resolution of a different kind. We compared, therefore, the patterns of plastid proteins in two-dimensional gel electrophoresis with isoelectric focusing in the first dimension and SDS-PAGE in the second (Fig. 5). The 35-kD polypeptide migrated as a single, homogeneous band, whereas the 58-kD polypeptide resolved into multiple spots which centered on isoelectric point = 6. These might correspond to discrete components or to a single species with minor charge modifications. In one-dimensional gels, the 58-kD polypeptide migrates near to that expected of the large subunit of ribulose-bisphosphate carboxylase. In isoelectric focusing, however, the large subunit has an isoelectric point of 5.85 (Fig. 5, right), distinctly more acidic than that of the 58-kD polypeptide.

Although the chromoplasts described here were all isolated from the variety Emerald Giant, we have also looked at chromoplasts from var Golden Cal Wonder, in which the immature fruits are green and the mature fruits are yellow, and var DNAP VS-12, in which the immature fruits are white or pale yellow and the mature fruits are red. We detected low levels of the 35- and 58-kD polypeptides in plastids from immature fruits of both Golden Cal Wonder and DNAP VS-12. In chromoplasts from these varieties, we found accumulation of the 35-kD polypeptide but none of the 58-kD species (data not shown).

**Quantitative Aspects.** The amounts of the 35- and 58-kD polypeptides in the chromoplasts estimated by densitometric scans of stained gels at 579 nm correspond to 10% and 30%, respectively, of total chromoplast protein.

## DISCUSSION

Analysis of the composition of chromoplasts starts as a problem in separation. Chromoplasts of daffodil can be recovered
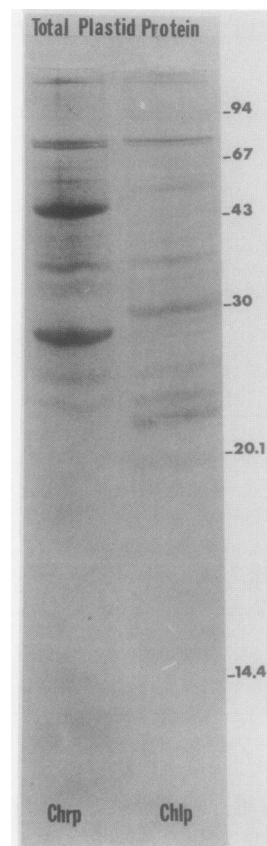


FIG. 4. SDS-PAGE of total chromoplast and chloroplast proteins in 5 M urea. In contrast to the pattern seen with non-urea gels (Fig. 3), no chloroplast polypeptides were detected co-migrating with the 58- and 35-kD polypeptides of chromoplasts.

from sucrose gradients (15). Tomato chromoplasts, however, are more fragile: they become leaky or disintegrate after sedimentation in gradients of sucrose or Percoll (1). Chromoplasts of *C. annuum* are more stable. Camara *et al.* (4) showed that intact chromoplasts of mature fruits could survive tissue disruption and differential centrifugation; we added a density gradient step to achieve better separation together with the recovery of intact chromoplasts.

We find that membranes of pepper chromoplasts contain high levels of 35- and 58-kD polypeptides, which are absent or present at very low levels in chloroplasts of green fruits. Similar proteins may occur in chromoplasts of other species but directly comparable data are rare. Beyer *et al.* (2) found moderately abundant polypeptides of approximately 35 and 58 kD in membranes of daffodil chromoplasts. Similar polypeptides can be seen in extracts of crude tomato chromoplasts (1), but they are not obviously enriched in gradient-purified organelles. Piechulla *et al.* (19) noted increases in a 35-kD membrane polypeptide and a 57-kD soluble polypeptide in total tissue extracts of tomato fruits.

Although we have no information on the function of the two chromoplast-specific proteins, one naturally looks to carotenoid-binding proteins (*cf.* Ref. 16) or to enzymes associated with carotenoid biosynthesis. D'Harlinque and Camara (7) isolated γ-tocopherol methyltransferase from chromoplast membranes of *C. annuum* and found it to have a mol wt of 33,000. This protein would be expected to occur in chloroplasts as well, but might conceivably be strongly induced in the chromoplasts.

Similarly, we can only speculate on the sites of synthesis of the 35- and 58-kD polypeptides. Since we detected no obvious chromoplast-specific transcripts from plastid DNA in *C. annuum* (11) nor incorporation of [$^{35}$S]Met into these polypeptides by
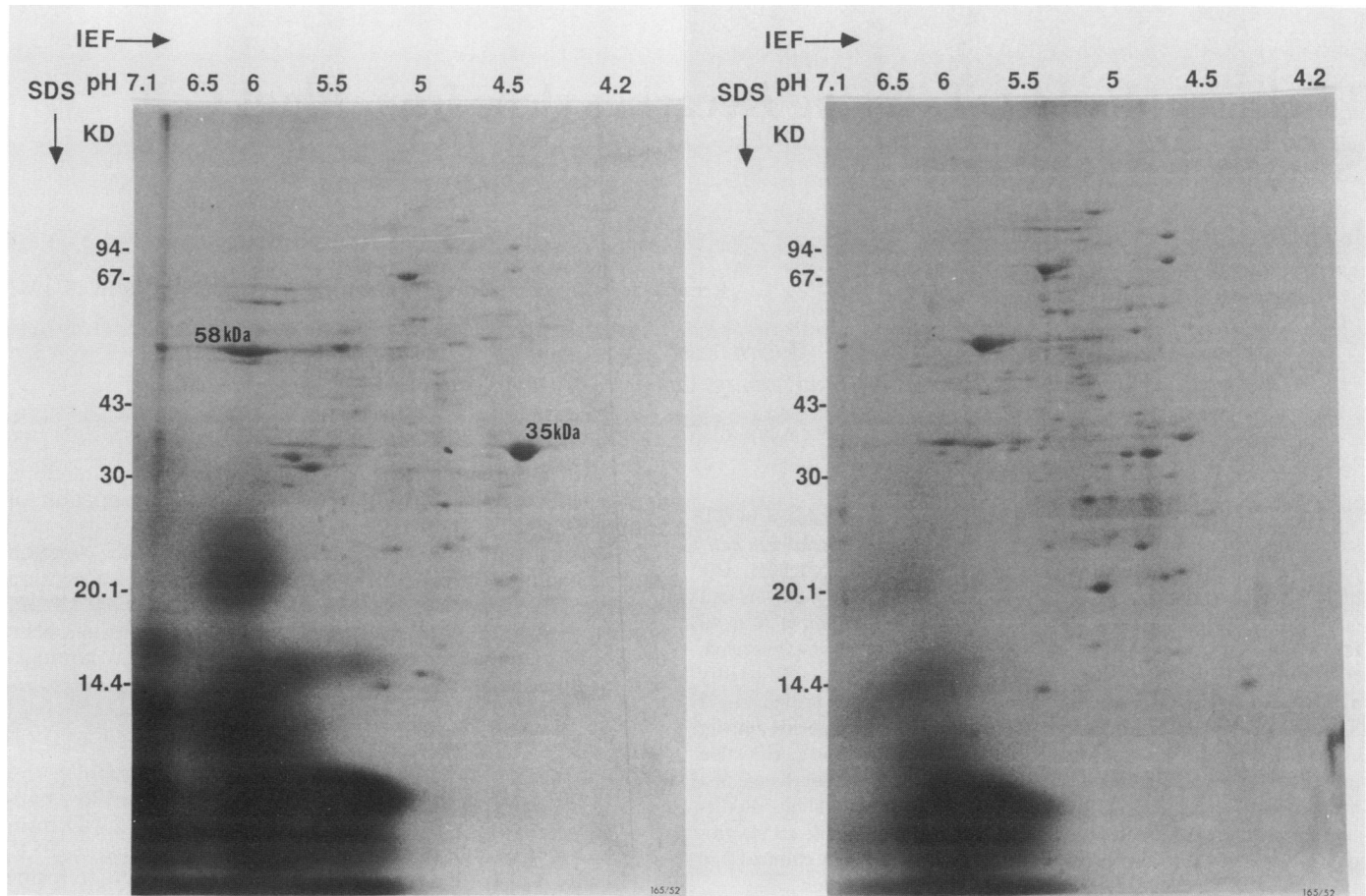
FIG. 5. Two-dimensional gel electrophoresis of proteins from chromoplasts and chloroplasts. Proteins were resolved by isoelectric focusing (IEF) in the first dimension and by SDS-PAGE in 9 to 15% w/v gradients in the second dimension. Left, chromoplast proteins; right, chloroplast proteins.

isolated chromoplasts (N Hadjeb, CA Price, unpublished data), we suspect that the proteins are coded in the nucleus and synthesized in the cytoplasm.

## LITERATURE CITED

1. BATHGATE B, ME PURTON, D GRIERSON, PW GOODENOUGH 1985 Plastid changes during the conversion of chloroplasts to chromoplasts in ripening tomatos. Planta 165: 197–204.
2. BEYER P, G WEISS, H KLEINIG 1985 Solubilization and reconstitution of the membrane-bound carotenogenic enzymes from daffodil chromoplasts. Eur J Biochem 153: 341–346
3. CAMARA B, J BRANGEON 1981 Carotenoid metabolism during chloroplast to chromoplast transformation in *Capsicum annuum* fruits. Planta 151: 359–364
4. CAMARA B, F BARDAT, O DOGBO, J BRANGEON, R MONEGER 1983 Terpenoid metabolism in plastids. Isolation and biochemical characteristics of *Capsicum annuum* chloroplasts. Plant Physiol 73: 94–99
5. CHUA N-H 1980 Electrophoretic analysis of chloroplast proteins. Methods Enzymol 69: 434–446
6. DAVIS BH, S MATTHEWS, JTO KIRK 1970 The nature and the biosynthesis of the carotenoids of different colour varieties of *Capsicum annuum*. Phytochemistry 9: 797–805
7. D'HARLINGUE A, B CAMARA 1985 Plastid enzymes of terpenoid biosynthesis: purification and characterisation of γ-tocopherol methyl transferase from *Capsicum* chloroplasts. J Biol Chem 260: 15200–15203
8. FREY-WYSSLING A, E KREUTZ 1958 The submicroscopic development of chromoplasts in fruits of *Capsicum annuum* L. J Ultrastr Res 1: 397–411
9. GIBBS M, JM ROBINSON 1974 Photosynthetic CO₂ incorporation in isolated spinach chloroplasts. *In* A San Pietro, ed, Experimental Plant Physiology, C. V. Mosby, St. Louis, pp 12–20
10. GIULANO G, D POLLOCK, PA SCOLNIK 1986 The gene *crtI* mediates the conversion of phytoene into colored carotenoids in *Rhodopseudomonas capsulata*. J Biol Chem 261: 12925–12929
11. GOUNARIS I, CA PRICE 1987 Plastid transcripts in chloroplasts and chromoplasts of *Capsicum annuum*. Current Genet 12: 219–224
12. HURTADO-HERNÁNDEZ H, PG SMITH 1985 Inheritance of mature fruit color in *Capsicum annuum* L. J Hered 86: 211–212
13. KORMOS J, K KORMOS 1960 Die Genetischen Typen der Carotenoid-Systeme der Paprikafrucht. Acta Bot 6: 305–319
14. LAEMMLI UK 1970 Cleavage of structural protein during the assembly of the head proteins of bacteriophage T4. Nature 227: 680–685
15. LIEDVOGEL B, P SITTE, H FALK 1976 Chromoplasts in the daffodil: fine structure and chemistry. Cytobiologie 11: 155–174
16. MASAMOTO K, HC RIETHMAN, LA SHERMAN 1987 Isolation and characterization of a carotenoid-associated thylakoid protein from the cyanobacterium *Anacystis nidulans* R2. Plant Physiol 84: 633–639
17. MILLER ME, JE JURGENSON, EM REARDON, CA PRICE 1983 Plastid translation *in organello* and *in vitro* during light-induced development in *Euglena*. J Biol Chem 258: 13478–13484
18. PICCIONI R, G BELLEMARE, N-H CHUA 1982 Methods of polyacrylamide gel electrophoresis in the analysis and preparation of plant polypeptides. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical, New York, pp 985–1013
19. PIECHULLA B, RE GLICK, H BAHL, A MELIS, W GRUISSEM 1987 Changes in photosynthetic capacity and photosynthetic protein pattern during tomato fruit ripening. Plant Physiol 84: 911–917
20. PRICE CA, JC CUSHMAN, LR MENDIOLA-MORGENTHALER, EM REARDON 1987 Isolation of plastids in density gradients of Percoll and other silica sols. Methods Enzymol 148: 157–179
21. ROSCOE TJ, RJ ELLIS 1982 Two dimensional gel electrophoresis of chloroplast proteins. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical, New York, pp 189–209
22. SPURR AR, WH HARRIS 1968 Ultrastructure of chloroplasts and chromoplasts in *Capsicum annuum* L. Thylakoid membrane changes during fruit ripening. Am J Bot 55: 1210–1224
23. WESTHOFF P, N NELSON, H BÜNEMANN, RG HERRMANN 1981 Localisation of genes for coupling factors subunits on the spinach plastid chromosome. Curr Genet 4: 109–110
24. WINKENBACH F, H FALK, B LIEDVOGEL, P SITTE 1976 Chromoplasts of *Tropaeolum majus* L.: isolation and characterization of lipoprotein elements. Planta 118: 23–33