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Isolation and Characterization of a Chromoplast-Specific Carotenoid-Associated Protein from *Cucumis sativus* Corollas[1]

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The differentiation of chloroplasts to chromoplasts in corollas of cucumber (*Cucumis sativus*) is subject to developmental control. To study factors involved in the chloroplast-chromoplast conversion, a chromoplast-specific protein of 35 kD was isolated, and polyclonal antibodies were prepared against it. This protein was found to be a principal component of the carotenoid-protein complex resolved from chromoplast membranes by nondenaturing gel electrophoresis. Immunological studies revealed that expression of this protein is regulated in a temporal and tissue-specific manner. Its steady-state level increased in parallel with flower development and carotenoid accumulation, peaking in mature flowers and then rapidly decreasing to very low levels. The protein was not detectable in cucumber leaves or fruits. To ascertain whether an organ-specific system regulates the chloroplast-chromoplast conversion and to enable future molecular studies of factors involved in this regulation, an in vitro bud culture system was established. Patterns of expression of the 35-kD protein and carotenoids in corollas of detached buds were similar to those in intact buds.

Most corollas are green during the early stages of flower development. Their subsequent coloration is due mainly to anthocyanin accumulation in the vacuoles and/or the accumulation of carotenoids in the chromoplasts. In some cases, mature pigmented corollas containing anthocyanin in the epidermal cell vacuoles also have been found to contain chloroplasts in the mesophyll (Weiss et al., 1988). Pathways of anthocyanin biosynthesis and their underlying molecular mechanisms have been widely studied (Van Tunen and Mol, 1990). Recently, some progress has been made toward understanding carotenoid biosynthesis in higher plants (Britton, 1989; Pecker et al., 1992; Pfander, 1992). However, the factors controlling chromoplast biogenesis and the molecular structure of this plastid are still largely unknown. The small amount of information available concerning the subject has come mainly from studies of fruits. The disappearance of Chl and the accumulation of carotenoids have been shown to parallel fruit maturation and ripening. At the same time, thylakoid membranes disintegrate, and most of the components of the photosynthetic machinery disappear. A new set of proteins accumulates instead, following the chloroplast-chromoplast conversion (Hansmann and Sitte, 1982, 1984; Bathgate et al., 1985; Hadjeb et al., 1988; Newman et al., 1989).

The chloroplast-chromoplast conversion in corollas has been shown to accompany flower development in cucumber (*Cucumis sativus*). In mature, yellow corollas, only chromoplasts could be found (Smith and Butler, 1971). To understand the structural organization of chromoplasts in corollas and to enable future characterization of the factors involved in the chloroplast-chromoplast conversion, we isolated a carotenoid-associated protein from *C. sativus* flowers. Immunological studies showed that expression of this protein is regulated in a temporal and tissue-specific manner.

MATERIALS AND METHODS

Plant Material

Cucumber (*Cucumis sativus*, cv Shimshon, obtained from Zeraim, Gedera, Israel) plants were grown under standard greenhouse conditions. Flower development was divided into five stages: Stages 1, 2, and 3 occurred 120, 72, and 24 h before anthesis, respectively; stage 4 occurred at anthesis; and stage 5 occurred 24 h after anthesis. Freshly collected buds were used in all experiments. Very young (stage 1) corollas were green and about 5 mm in length.

Plastid Isolation

All chromoplast isolation steps were performed at 4°C, essentially as described by Bathgate et al. (1986). Briefly, 50 g of corollas were collected at anthesis and were cut with a razor blade into 3- × 3-mm segments. The segments were ground in a razor-blade blender (three 1-s pulses) in 500 mL of buffer containing 400 mM Suc, 2 mM $MgCl_2$, 10 mM KCl, 8 mM EDTA, and 100 mM Tris-HCl (pH 8.2). The suspension was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 5000*g* for 10 min. The pellet was resuspended in the same buffer, and chromoplasts were purified by centrifugation for 1 h at 74,000*g* on a discontinuous Suc gradient (15, 30, 40, and 50%, w/v). Chromoplasts collected from the 30 to 40% interface were diluted to bring the Suc concentration to 400 mM and pelleted for 5 min at 5000*g*. The chromoplast preparation was monitored for purity and intactness by light microscopy following each isola-

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Abbreviation: LHCII, the major light-harvesting Chl *a*/*b*-binding protein.

tion step. Chloroplasts were prepared from cucumber leaves as described previously (Bathgate et al., 1985).

In Vitro Culture

Very young flower buds (stage 1) were collected, rinsed several times with sterile water, and placed in a culture vessel containing 150 mM Suc. Inside the vessels, buds were placed on a floating stand, such that only their bases were in contact with the culture media. The buds were cultured for the specified periods under constant light from cool-white fluorescent lamps (80 $\mu E\ s^{-1}m^{-2}$, Tadiran, Tel Aviv, Israel) at 24°C.

SDS-PAGE and Protein Transfer to Nitrocellulose Filters

Proteins were extracted and precipitated with TCA as described by Bathgate et al. (1986). Electrophoresis of the proteins on an SDS-polyacrylamide gel, either 12 or 10 to 15% linear gradient containing urea, was performed according to the procedure of Laemmli (1970) or Thornber et al. (1986), respectively. Resolved proteins were either stained with Coomassie brilliant blue or electrotransferred to nitrocellulose filters (Schleicher & Schuell) as described by Towbin et al. (1979) and detected with specific antibodies and ^{125}I-protein A.

Separation and Analysis of Pigment-Protein Complexes

Chromoplasts were washed with 2 M NaBr and solubilized at 4°C or at 20°C for up to 30 min with various detergent combinations. The best resolution of the pigmented complex was obtained when solubilization was performed with 2, 1, and 0.3% or with 1, 2, and 0.3% (v/v) octyl glucoside, nonyl glucoside, and SDS, respectively, for 15 min at 20°C. Electrophoresis on nondenaturing Deriphat-polyacrylamide gels (4 and 5%) at 100 V for up to 60 min was performed as described by Peter and Thornber (1991). An entire lane containing pigmented bands or a single band was then excised from this gel and reelectrophoresed on the fully denaturing, 10 to 15% SDS-polyacrylamide gel described above (Thornber et al., 1986).

Immunofluorescent Labeling

Chromoplasts or chloroplasts were placed on slides and fixed for 30 min at 4°C, first in ethanol:acetic acid (1:3, v/v), then in 70% ethanol, and finally in 100% ethanol. Following a wash in 0.1 M potassium phosphate buffer, plastids were incubated with specific affinity-purified antibodies or with the buffer and preimmune serum as a control. After the plastids were rinsed three times in the same buffer, they were incubated with rhodamine-labeled goat anti-rabbit antisera. Following three more rinses as above, plastids were examined in an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) and photographed. Optimal conditions were established by probing chromoplasts and chloroplasts with antibodies to LHCII (Broido et al., 1991).

Analytical Methods

Carotenoid content was determined as described by Goodwin (1965). Protein content was determined according to the method of Lowry et al. (1951). To prepare antibodies against the 35-kD protein, purified chromoplasts were electrophoresed on a 10 to 15% SDS-polyacrylamide gel, the band containing the specific protein was excised, and the protein was eluted in a sample concentrator (ISCO) (Hunkapiller et al., 1983). Preparation of polyclonal antibodies in rabbit and affinity purification using nitrocellulose filters were as described by Bulinski and Borisy (1980) and Smith and Fisher (1984), respectively. EM was performed as described previously (Weiss et al., 1990). All experiments were repeated at least four times.

RESULTS

Isolation of a Tissue-Specific, Carotenoid-Associated Protein from Cucumber Corollas

The protein composition of *C. sativus* chromoplasts, obtained from yellow corollas at anthesis (stage 4), was analyzed by SDS-PAGE and compared to that of leaf chloroplasts. Several polypeptides that were clearly detectable in the total protein fraction of chromoplasts were not detectable in chloroplasts and vice versa (Fig. 1). A distinct chromoplast-specific polypeptide of 35 kD (estimated from 12% SDS-PAGE) was isolated. Its mobility on a urea-containing 10 to 15% linear gradient SDS-polyacrylamide gel corresponded to a molecular mass of about 33 kD (Fig. 1). Antibodies prepared against this polypeptide cross-reacted with the 35-kD chromoplast polypeptide (Fig. 1, lane 6), whereas no cross-reactivity with chloroplast proteins was detected. The antibodies were shown to be tissue specific, because they did not cross-react with the total protein fraction of cucumber leaves or fruits, whereas a strong signal was obtained with corollas (Fig. 1).

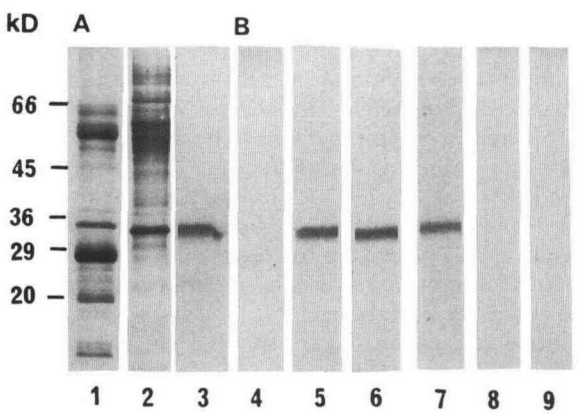

Figure 1. Isolation and immunodecoration of the 35-kD protein. Total protein from cucumber chloroplasts (20 μg, lanes 1 and 4), chromoplasts (20 μg, lanes 2 and 5), mature corollas (30 μg, lane 7), leaves (30 μg, lane 8), and fruits (30 μg, lane 9) and purified 35-kD protein (2 μg, lanes 3 and 6) were resolved by urea-containing 10 to 15% linear gradient SDS-PAGE and either stained with Coomassie brilliant blue (A) or analyzed by western blotting (B) using antibodies against the 35-kD protein and ^{125}I-protein A.

Cross-reactivity was also detected with squash, watermelon, and melon corollas, whereas none could be detected with their respective fruits or with tomato and pepper fruits. It should also be noted that the preimmune serum did not reveal any signal when used in a western blot analysis of corollas, chromoplasts, or purified 35-kD protein (not shown).

Immunofluorescence staining was used to confirm the localization of the antigen in the chromoplasts (Fig. 2). Fluorescence microscopy revealed the presence of distinct fluorescent signals when chromoplasts were incubated with affinity-purified antibodies to the 35-kD polypeptide. Using preimmune serum (or buffer, not shown) as a control, we determined that the fluorescence was specific to the anti-35-kD antibodies rather than being due to nonspecific binding of the anti-rabbit antibodies or to autofluorescence of the tissue (Fig. 2).

To examine possible relationships between the 35-kD protein and chromoplast pigments, plastid components were fractionated on a nondenaturing gel (Fig. 3). It should be noted that chromoplast membranes were washed with NaBr

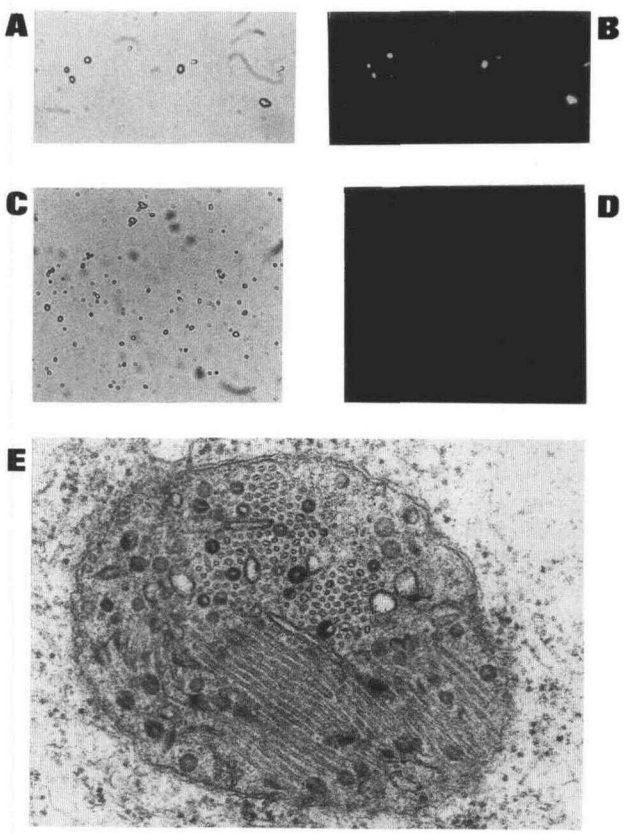

Figure 2. Fluorescent immunolabeling of the 35-kD protein. Immunofluorescent staining of the 35-kD protein was performed on isolated, fixed chromoplasts using antibodies against the 35-kD protein (A and B) or preimmune serum as a control (C and D), followed by labeling with rhodamine-conjugated immunoglobulin. E, EM of a thin section of corolla chromoplast (×68,000). A and C, Transmitted-light phase contrast microscopy; B and D, reflected-light fluorescence microscopy (A–D, ×230).

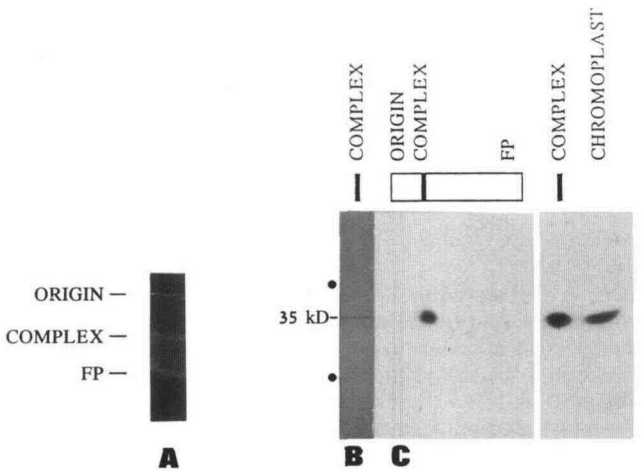

Figure 3. Identification of the 35-kD protein as a carotenoid-associated protein in chromoplast membranes using two-dimensional gel electrophoresis. NaBr-washed chromoplast membranes isolated from corollas at anthesis were solubilized with 2% octyl glucoside, 1% nonyl glucoside, and 0.3% SDS for 15 min at 20°C and fractionated on a nondenaturing Deriphat-polyacrylamide (5%) gel for 30 min at 100 V. The gel was photographed without fixing or staining (A). One entire lane and an upper yellow band (complex) were excised from the nondenaturing gel and reelectrophoresed on a fully denaturing 10 to 15% linear gradient SDS-polyacrylamide gel. For comparison, purified chromoplasts were also analyzed. B, Coomassie brilliant blue staining of the reelectrophoresed complex. C, Western blotting using antibodies against the 35-kD protein and ^{125}I-protein A. FP, Free pigment zone.

before solubilization with detergents, and two yellow bands were apparent. A discrete, slow-migrating band was revealed following solubilization by either of the two detergent combinations described in "Materials and Methods." The migration rate and abundance of the lower, diffuse band (presumably containing free pigment) varied with the different solubilization conditions. Second-dimension gel electrophoresis under fully denaturing conditions, followed by Coomassie staining and western blot analysis, revealed the 35-kD protein to be associated with the upper, slow-migrating pigmented band (Fig. 3). In some experiments, two additional minor proteins overlapping with the upper pigmented band were detected by Coomassie staining of the second-dimension gel (indicated by dots in Fig. 3B). As expected, the fast-migrating band did not contain any detectable amounts of protein. Although mobility of the upper, slow-migrating yellow band was not identical under the two solubilization conditions used, the 35-kD protein was found to comigrate with this discrete band under both solubilization conditions. Length of electrophoresis (up to 60 min) had no effect on this tight association. In contrast, boiling the chromoplast membranes or increasing the solubilizing detergent concentrations abolished this association.

Kinetics of the Accumulation of 35-kD Protein and Carotenoids in Relation to Flower Bud Development in Vivo and in Vitro

Expression of the 35-kD protein in corollas was controlled in a temporal manner (Fig. 4). Corolla development in vivo

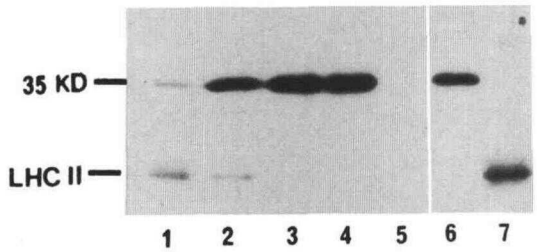

Figure 4. Changes in the immunologically detectable amounts of 35-kD protein and LHCII during flower development in vivo. Total protein (30 μg of protein per lane) extracted from corollas at stages 1 through 5 (lanes 1–5, respectively), isolated chromoplast total protein (5 μg, lane 6), and leaf total protein (30 μg, lane 7) were electrophoresed on a urea-containing 10 to 15% linear gradient SDS-polyacrylamide gel. Western blot analysis was performed using antibodies against the 35-kD protein and then against LHCII.

was accompanied by accumulation of the 35-kD protein. Its level (per unit protein) peaked in mature, stage-3 corollas and then remained nearly constant until anthesis (stage 4). Thereafter, the level of the protein rapidly decreased and was barely detectable at stage 5 (24 h postanthesis). The pattern of LHCII expression (a chloroplast protein) during flower development was characteristic of tissue in which chloroplast-chromoplast conversion is occurring. Its amount in corollas gradually decreased, in parallel with corolla development, reaching undetectable levels in mature corollas (Fig. 4).

Figures 5 and 6 show that corolla development in vitro, like flower development in vivo, was accompanied by an increase in the content of the 35-kD protein and carotenoids, respectively. However, the patterns of their respective accumulations differed. After only 1 d of culture, the steady-state level of the 35-kD protein had increased sharply (Fig. 5). In contrast, carotenoid content remained at an almost constant

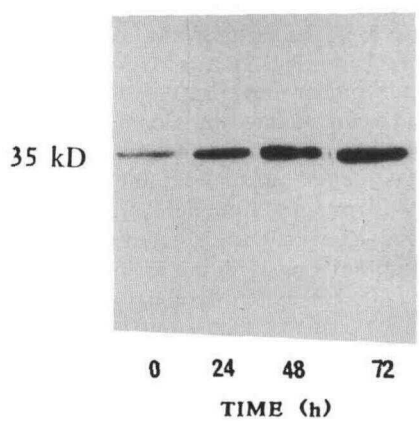

Figure 5. Changes in the immunologically detectable amounts of 35-kD protein during flower development in vitro. Very young flower buds (stage 1) were cultured on Suc-containing media for 24, 48, and 72 h. Total protein (30 μg) was extracted from corollas and, following electrophoresis on a urea-containing 10 to 15% linear gradient SDS-polyacrylamide gel, was analyzed by western blotting.

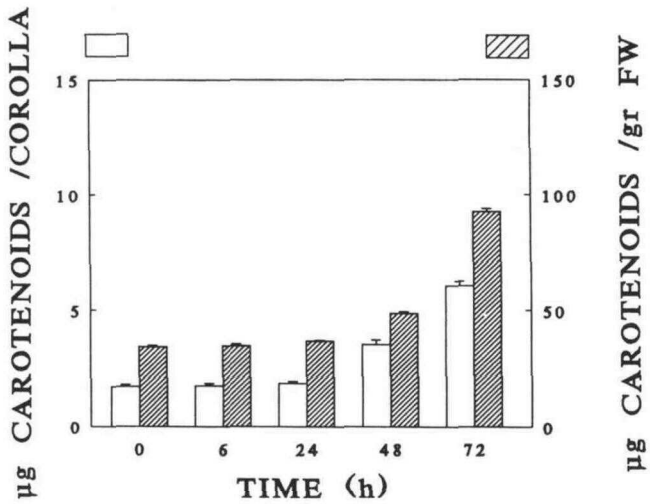

Figure 6. Changes in the carotenoid content of corollas during flower development in vitro. Very young flower buds (stage 1) were cultured for different periods in Suc media. Carotenoid content of corollas was measured as described in "Materials and Methods." FW, Fresh weight; bars, SE.

level during the 1st d of culture. During the next 2 d, however, carotenoid content per bud increased 3.5-fold. When calculated per unit fresh weight, the increase in carotenoid content was somewhat smaller (about 2.5-fold), probably indicating that carotenoid synthesis in vitro lags behind corolla growth (Fig. 6).

DISCUSSION

EM studies have established the presence of chloroplasts in the corollas of young green *C. sativus* flowers, as well as their conversion to chromoplasts as the flower matures (Smith and Butler, 1971). These corollas, therefore, provide a useful system to study chloroplast-chromoplast conversion during flower development. An abundant chromoplast 35-kD protein, undetectable in chloroplasts, was purified, and polyclonal antibodies were prepared against it. No contaminating protein was detected when the N-terminal amino acid sequence of the purified antigen was analyzed, indicating the use of a single polypeptide in the antibody preparation (not shown). Immunochemical studies revealed the expression of the antigen to be regulated in a temporal and tissue-specific manner. The possibility that the 35-kD protein was undetectable in leaves or chloroplasts because of interference from high amounts of Rubisco can be ruled out, because neither overloading of the gels nor overexposure of the autoradiograms revealed a specific signal in these samples (not shown). The steady-state level of the protein in corollas increased in parallel with flower development, the latter being known to be accompanied by chromoplast accumulation (Smith and Butler, 1971), and peaked in mature flowers. Both pattern of expression and immunofluorescent studies strongly indicated that the antigen was a chromoplast component.

Chromoplast-specific proteins have been described previ-

ously for pepper (Hadjeb et al., 1988; Newman et al., 1989) and tomato (Bathgate et al., 1985) fruits and daffodil (Hansmann and Sitte, 1984) and violet (Hansmann and Sitte, 1982) flowers. The molecular masses of the chromoplast-specific proteins in pepper fruits were found to be about 58 and 35 kD. Even though the mobility of the protein that we purified was similar to one of those in pepper, these proteins are most probably not identical. This assumption is based on their pattern of expression and on the observation that antibodies prepared during the course of the present study did not cross-react with plastids from any of the above-mentioned fruits. Furthermore, the mobility of our 35-kD protein on a urea-containing SDS-polyacrylamide gel differed from that reported for the chromoplast-specific pepper proteins (Hadjeb et al., 1988). Finally, no homology could be found between 20 N-terminal amino acids of the *C. sativus* protein and recently described sequences of chromoplast-specific pepper proteins (Hadjeb et al., 1991) or any other sequence available in GenBank (not shown). Nevertheless, full sequences of these proteins are needed before their relatedness can be categorically established.

Carotenoids are known to be associated with the Chl-protein complexes of thylakoid membranes in chloroplasts (Peter and Thornber, 1991). Recently, steps toward understanding the organization of carotenoids in higher plant chromoplasts have been taken: a 58-kD protein was found to be associated with the pigmented complex in pepper fruits (Cervantes-Cervantes et al., 1990), and a carotenoprotein complex was isolated from carrots (Milicua et al., 1991). Results of the present study strongly indicate that the 35-kD protein is a component of the pigmented complex in cucumber corolla chromoplasts. At present, we are unable to determine whether the 35-kD protein is actually a carotenoid-binding protein or a pigmentless polypeptide that is part of the pigmented complex. Because the protein comigrated with the pigmented complex and was a major polypeptide in that complex under various solubilization conditions, we suggest that it could, in fact, be an apoprotein.

Flower development in vivo is controlled by a complex array of regulatory factors that involve tight interaction between the bud and the rest of the plant (Kinet et al., 1985). To ascertain whether an organ-specific system is regulating chloroplast-chromoplast conversion and to study factors involved in this regulation, an in vitro bud culture system was used. We showed that the patterns of expression of the 35-kD protein and the carotenoids in corollas of detached buds (Figs. 5 and 6) were similar to those in intact buds (Fig. 4; Vainstein et al., 1992). In both systems, accumulation of the 35-kD protein and carotenoids correlated well with flower development.

Dissection of the mechanism(s) controlling this protein's expression and a determination of its precise role in corolla chromoplasts should contribute to an elucidation of the processes involved in chromoplast biogenesis and carotenoid biosynthesis.

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