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Isolation and Regulation of Accumulation of a Minor Chromoplast-Specific Protein from Cucumber Corollas[1]

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The differentiation of chloroplasts to chromoplasts in cucumber (*Cucumis sativus* L.) corollas parallels flower development. Chromoplast biogenesis involves chlorophyll degradation, carotenoid accumulation, and the appearance of a new set of proteins. To study factors involved in chromoplast biogenesis in floral tissues, a minor (in abundance) protein of about 14 kD, CHRD (chromoplast protein D), was isolated from cucumber corolla chromoplasts. Immunological characterization revealed that the protein is chromoplast-specific and that its steady-state level in corollas increases in parallel to flower development. The protein was not detected in cucumber leaves or fruits. Immunological analysis of corollas and fruits from a variety of other plants also did not reveal cross-reactivity with the CHRD protein antisera. Using an in vitro bud culture system, we analyzed the effect of phytohormones on CHRD expression. Gibberellic acid rapidly enhanced, whereas paclobutrazol down-regulated, the steady-state level of CHRD. Ethylene also down-regulated the protein's steady-state level. It is suggested that hormonal control of chromoplastogenesis is tightly regulated at the tissue/organ level and that mainly developmental signals control carotenoid accumulation in nonphotosynthetic tissues.

Carotenoids serve several important functions in a variety of organisms. In plants they accumulate mainly in chloroplasts and chromoplasts, serving in the former as a very efficient antioxidant. In the past decade advances have been made in our understanding of the carotenoid biosynthetic pathway at the molecular level. A number of higher plant genes from this pathway have been cloned and characterized (Bartley and Scolnik, 1995). However, much less information is available about carotenoid accumulation in chromoplasts, which dictate the color of a variety of fruits and flowers. With the exception of capsanthin-capsorubin synthase (Bouvier et al., 1994; Houlne et al., 1994), to our knowledge chromoplast-specific expression has not been shown for any of the genes cloned to date from the carotenoid biosynthetic pathway (Bartley and Scolnik, 1995), despite the fact that carotenoids composition and their structural organization within chromoplasts are unique to this organelle (Emter et al., 1990; Fraser et al., 1994).

Studies of chromoplast biogenesis have revealed that chlorophyll degradation, carotenoid accumulation, and the appearance of a new set of proteins parallel fruit or flower development (Newman et al., 1989; Emter et al., 1990; Marano et al., 1993; Oren-Shamir et al., 1993; Bouvier et al., 1994; Deruere et al., 1994; Bartley and Scolnik, 1995). To date, only a handful of chromoplast-specific proteins have been isolated and immunologically characterized (Bartley and Scolnik, 1995), and all of these are abundant proteins that, aside from the exception mentioned above, are most probably involved in the structural organization of the carotenoids. One such chromoplast-specific, carotenoid-associated protein from pepper fruits, termed fibrillin, was recently cloned (Deruere et al., 1994). Characterization of this gene's expression revealed that fibrillin's accumulation is regulated mainly at the RNA level in a temporal and tissue-specific manner (Deruere et al., 1994). A similar accumulation pattern and regulation level have also been revealed for a carotenoid-associated chromoplast protein, CHRC, from *Cucumis sativus* corollas (Vainstein et al., 1995; Vishnevetsky et al., 1996).

Analysis of the regulation of chromoplastogenesis has revealed that the absence of light, an environmental signal, leads to the promotion of chloroplast-chromoplast conversion in both fruits and corollas (Goldschmidt, 1988; Boyer, 1989; Vainstein et al., 1994). In contrast, the effect of hormones on chromoplastogenesis in fruits is opposite that in floral tissues. In the former, ethylene promotes chloroplast-chromoplast conversion and GA_3 delays it (Thomson et al., 1967; Goldschmidt, 1988); in *C. sativus* corollas, ethylene delays and GA_3 promotes chromoplast biogenesis (Vainstein et al., 1994). In contrast to fibrillin, which in pepper fruits is down-regulated by GA_3 and up-regulated by ABA (Deruere et al., 1994), the level of CHRC in cucumber corollas is strongly up-regulated by GA_3 and down-regulated by ABA (Vainstein et al., 1994). The specific up-regulation of CHRC by GA_3 in cucumber corollas is very rapid and this hormone is proposed to be involved in the regulation of carotenoid accumulation during flower development (Bartley and Scolnik, 1995). To gain further insight into chromoplast buildup in floral tissues, a novel minor (in abundance) chromoplast-specific protein of 14

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Abbreviations: CHRC, carotenoid-associated chromoplast protein; CHRD, chromoplast protein D.

kD (hereafter designated CHRD) was isolated from *C. sativus* corollas, immunologically characterized, and analyzed in terms of the regulation of its expression by developmental and environmental signals.

MATERIALS AND METHODS

Cucumis sativus L. cv Shimshon plants, obtained from Zeraim Gedera (Gedera, Israel), were grown under standard greenhouse conditions. Flower development was categorized into five stages: stages 1, 2, and 3 represent flowers 120, 72, and 24 h before anthesis, respectively; stage 4 represents flowers at anthesis; and stage 5 represents flowers 24 h after anthesis. For etiolation experiments, stage 1 flowers were enclosed in aluminum foil for 3 d.

Plastid Isolation and Analysis of Pigment-Protein Complexes

Chromoplasts were isolated as described by Smirra et al. (1993). Membranes were solubilized at 20°C for 15 min with the following detergent combinations: 2, 1, and 0.3% or 1, 2, and 0.3% (w/v) *n*-octyl α-D-glucopyranoside (octyl glucoside), *n*-nonyl α-D-glucopyranoside (nonyl glucoside), and SDS, respectively; 2, 1, 1, and 0.3%, 1, 1, 2, and 0.3%, or 1, 2, 1, and 0.3% (w/v) octyl glucoside, nonyl glucoside, *n*-decyl α-D-glucopyranoside (decyl glucoside), and SDS, respectively; or the same combinations, substituting Triton X-100 for decyl glucoside. Electrophoresis on nondenaturing Deriphat-polyacrylamide gels (5%) at 100 V for 20 to 40 min was performed as described by Peter and Thornber (1991). An entire lane containing pigmented bands was then excised from this gel and sliced. Gel slices were frozen at −20°C overnight and then ground in 100 mM Tris, pH 7.5. After the sample was centrifuged (15,000*g*, 5 min) the pellet was discarded, and proteins that had been precipitated from the supernatant with TCA were resuspended in Laemmli sample buffer (Laemmli, 1970).

SDS-PAGE and Western Blotting Analysis

SDS-PAGE of the extracted and total corolla proteins and western blot analysis using affinity-purified polyclonal antibodies against CHRD and CHRC and alkaline phosphatase anti-rabbit IgG were performed as described by Smirra et al. (1993). When indicated, the western blot analyses were performed with an enhanced chemiluminescence system (ECL, Amersham), using affinity-purified polyclonal antibodies against CHRD and horseradish peroxidase anti-rabbit IgG (Sigma) as a secondary antibody. Autoradiogram quantification was carried out by scanning appropriately exposed films in a densitometer (Molecular Dynamics, Sunnyvale, CA). Each experiment was repeated at least three times with three amounts of protein (10, 25, and 50 μg per lane) for each sample.

In Vitro Culture

Stage 1 flower buds were collected and rinsed several times with sterile water. Buds were placed on a perforated Parafilm (American National Can, Greenwich, CT) covering a Petri dish filled with double-distilled sterile water such that only the bases of the buds were in contact with the liquid. When indicated, GA_3 (100 μM), ABA (100 μM), or paclobutrazol (100 μg mL^{-1}) was added to the double-distilled sterile water, or ethylene (10 parts per million) was injected into a vessel containing the Petri dish. Buds were cultured for the specified periods at 23°C, under constant light from cool-white fluorescent lamps with a PPFD of 80 μmol m^{-2} s^{-1}.

Analytical Methods

Protein content was determined according to the method of Bradford (1976). To prepare antibodies against CHRD, purified chromoplasts were electrophoresed on a 10 to 15% SDS-polyacrylamide gel, and the band containing the specific minor protein was excised. Affinity-purified rabbit polyclonal antibodies against CHRD were prepared as described previously for CHRC (Smirra et al., 1993).

RESULTS

Characterization of CHRD

The polypeptide compositions of chromoplasts, chloroplasts, and corollas at different developmental stages were compared. One of the polypeptides, the 14-kD protein CHRD, was present in chromoplasts, undetectable in chloroplasts, and accumulated in corollas in parallel with flower development (as determined by Coomassie brilliant blue staining). CHRD is a minor protein (Fig. 1): based on densitometry analyses it constitutes about 1% of the total chromoplast proteins resolved by SDS-PAGE. The protein was isolated and used to prepare antiserum, and the resultant antiserum cross-reacted with isolated CHRD and did not cross-react with CHRC or chloroplasts. Preimmune serum did not reveal any signal when used in western blot analysis of corollas, chromoplasts, or isolated CHRD. The

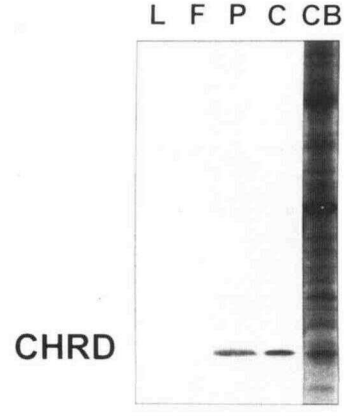

Figure 1. Immunodecoration of CHRD in different *C. sativus* organs. Total protein extracted from leaf (L), fruit (F), corolla (P) (50 μg per lane), and chromoplasts (C) (10 μg of protein) were electrophoresed on a 14% SDS-polyacrylamide gel and analyzed by western blotting using antibodies against CHRD and alkaline phosphatase anti-rabbit IgG. CB, Coomassie brilliant blue-stained total proteins (25 μg) from chromoplasts.

affinity-purified antibodies against CHRD were found to be tissue-specific: they did not cross-react with the total protein fraction of cucumber leaves or fruits, whereas a strong signal was obtained with corollas and isolated chromoplasts (Fig. 1). During flower development the immunologically detectable level of CHRD increased in corollas up to anthesis and then decreased to a low level (Fig. 2).

To examine the possible relationship between CHRD and chromoplast pigments, the plastids were solubilized with various detergent combinations and fractionated on a nondenaturing gel (Fig. 3). A distinct, slow-migrating, yellow band was revealed under the following solubilization conditions: 2, 1, 1, and 0.3%; 1, 1, 2, and 0.3%; or 1, 2, 1, and 0.3% (w/v) octyl glucoside, nonyl glucoside, decyl glucoside, and SDS, respectively. Western blot analysis of the polypeptides extracted from this distinct band and resolved by fully denaturing SDS-PAGE revealed the presence of CHRD and CHRC (Fig. 3). As expected (Smirra et al., 1993), Coomassie brilliant blue staining of the SDS gels revealed CHRC to be the major polypeptide associated with these yellow bands.

To test for CHRD antigenic counterparts in chromoplastogenic organs of other plants, CHRD antibodies were cross-reacted with fruits and petals of a variety of different plants. Unlike CHRC, which is abundant in several other flowers (Smirra et al., 1993), CHRD antiserum did not cross-react with corollas of melon, watermelon, daffodil, or rose, or with fruits of pepper or tomato, or with carrot.

Effects of Developmental and Environmental Factors on CHRD Accumulation

Previous studies have shown the usefulness of the in vitro bud culture system, which fully resembles flower development with respect to carotenoid and CHRC accumulation, for studies of the involvement of growth regulators in chromoplast biogenesis (Vainstein et al., 1994). We used this system to study the developmental regulation of CHRD expression in corollas. Inclusion of GA_3 in the culture medium resulted in enhanced accumulation of CHRD (Fig. 4A). When paclobutrazol, an inhibitor of GA_3 synthesis, was added to the in vitro bud culture system, the CHRD level per unit protein was down-regulated 4.8 ± 0.2 times as compared with control untreated corollas. A lower level of CHRD (although the decrease was less pronounced) was also detected in corollas treated with ABA, which is known to be antagonistic to GA_3 in several systems (Pharis and King, 1985; Huttly and Baulcombe, 1989; Shi et al., 1992) (2.5 ± 0.3 times lower than that of control untreated corollas per unit protein) (Fig. 4A). The down-regulation–up-regulation of CHRD by GA_3 was very rapid, and after only 2 h its level was markedly higher in treated versus nontreated buds (Fig. 4B). No difference was noted in fresh weight or total protein level between treated and control corollas for up to 24 h in culture.

The effect of ethylene, a growth regulator associated with fruit ripening and flower senescence (Trebitsh et al., 1993), on the accumulation of chromoplast-specific proteins is shown in Figure 5A. The level of CHRD per unit protein was down-regulated following treatment with ethylene. Moreover, the level of CHRC in ethylene-treated corollas was also lower than that in controls.

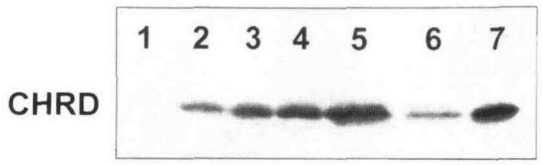

Figure 2. Changes in the immunologically detectable amounts of CHRD during flower development in vivo. Total protein (50 μg per lane) extracted from leaf (lane 1), corollas at stages 1 through 5 (lanes 2–6, respectively), and chromoplasts (10 μg, lane 7) was electrophoresed on a 14% SDS-polyacrylamide gel and analyzed by western blotting.

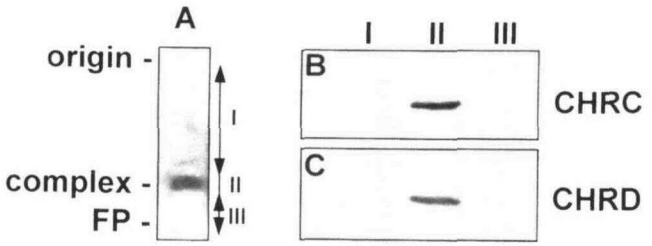

Figure 3. Identification of CHRD in the carotenoid-protein complex of chromoplasts. Chromoplast membranes isolated from corollas at anthesis were solubilized with 2% *n*-octyl α-D-glucopyranoside, 1% *n*-nonyl α-D-glucopyranoside, 1% *n*-decyl α-D-glucopyranoside, and 0.3% SDS (w/v) and fractionated on a nondenaturing Deriphat-polyacrylamide gel for 30 min at 100 V. The gel was photographed without fixing or staining (A). The entire lane was excised from the gel and cut into three pieces (lanes I–III). Proteins were extracted from these gel slices, re-electrophoresed on a fully denaturing 14% SDS-polyacrylamide gel, and analyzed by western blotting using CHRC (B) and CHRD (C) antibodies. FP, Free pigment zone.

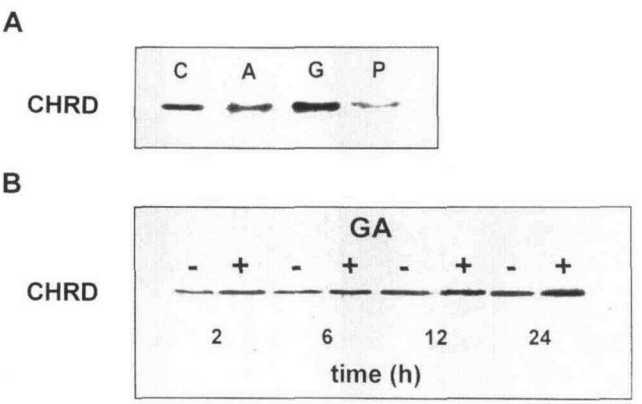

Figure 4. Effect of GA_3 on the CHRD content of in vitro cultured corollas. A, Young flower buds were cultured for 72 h in the presence of GA_3 (G), ABA (A), or paclobutrazol (P) or without phytohormones (C), and CHRD levels in corollas were analyzed by western blotting (10 μg of total protein per lane) using antibodies against CHRD and horseradish peroxidase anti-rabbit IgG in an enhanced chemiluminescence detection system. B, Western blot analysis of corollas (50 μg of total protein per lane) cultured for the indicated periods with (+) or without (−) GA_3. The blot was decorated with antibodies against CHRD and alkaline phosphatase anti-rabbit IgG.

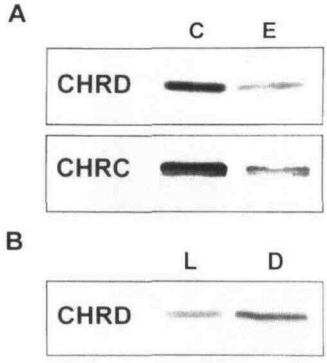

Figure 5. Effect of ethylene on CHRC and CHRD levels and of etiolation on CHRD levels in corollas. A, Antibodies against CHRD or CHRC were used in a western blot analysis of CHRD and CHRC levels, respectively, in corollas cultured for 72 h in the presence (E) or absence (C) of ethylene (50 μg of total protein per lane). B, Flowers were enclosed in aluminum foil in the greenhouse for 3 d and CHRD levels in etiolated (D) and control, nonetiolated (L) corollas were analyzed by western blotting (50 μg of total protein per lane).

Carotenoids, in contrast to chlorophyll, also accumulate in the dark. To monitor the effect of light, CHRD levels in in vivo etiolated corollas were analyzed. Figure 5B shows the CHRD level per unit protein in corollas of etiolated buds to be enhanced as compared with that in control corollas of light-grown buds. It is worth noting that effects of both ethylene and etiolation on CHRD levels normalized per corolla were even more pronounced, since the total protein content of the treated corollas was, respectively, 50% lower and 30% higher than that of control, untreated corollas.

DISCUSSION

Corollas of *C. sativus* flowers are green during the early stages of flower development. Coloration at later stages of development is due to the accumulation of carotenoids in the chromoplasts (Vainstein et al., 1994). Electron microscopy studies have established the presence of chloroplasts in the corollas of young, green flowers, as well as their conversion to tubulous chromoplasts as the flower matures. Only chromoplasts are found in the mature, yellow flower corollas (Smith and Butler, 1971; Smirra et al., 1993; Vainstein et al., 1994). GA_3, a developmental signal, was suggested to be involved in the regulation of chromoplastogenesis in corollas, based mainly on an immunological characterization of the regulation of CHRC expression (Bartley and Scolnik, 1995).

To further delineate chromoplast biogenesis in floral tissue, CHRD was isolated and immunologically characterized. As with CHRC (Vainstein et al., 1994), the level of this chromoplast-specific protein was found to be rapidly (on the order of hours) up-regulated by GA_3. Carotenoids also accumulate to a higher level in GA_3-treated versus control corollas, albeit at a slower rate (Vainstein et al., 1994). Moreover, recently GA_3 has been shown to up-regulate the steady-state level of CHRC transcript in a matter of minutes (Vainstein et al., 1995). Taken together, the available data indicate that in floral tissue pigmentation due to carotenoid accumulation, like that due to anthocyanin accumulation (Weiss et al., 1992, 1993; Martin and Gerats, 1993), is under tight, positive control by GA_3.

In contrast to floral tissue, GA_3 in fruits delays chromoplastogenesis (Coggins and Lewis, 1962; Goldschmidt, 1988), and based on studies with fibrillin, it down-regulates the level of carotenoid-associated protein (Deruere et al., 1994). The growth regulator ethylene promotes chloroplast-chromoplast conversion in climacteric fruits (Goldschmidt, 1988), whereas in corollas it down-regulates the levels of both CHRC and CHRD. Hence, the hormonal control of chromoplastogenesis is suggested to be tightly regulated at the organ/tissue level.

The biogenesis of chloroplasts and the accumulation of apoproteins in these plastids require light (Terzaghi and Cashmore, 1995). On the other hand, etiolation promotes chromoplastogenesis in *C. sativus* corollas and elevates the levels of CHRC (Vainstein et al., 1994) and CHRD (Fig. 5). The data concerning environmental control, together with those concerning the developmental control of chromoplast biogenesis and CHRC and CHRD expression, strongly support the model proposed by Bartley and Scolnik (1995), in which mainly developmental signals control carotenoid accumulation in nonphotosynthetic tissues.

In the present study we characterized a minor protein that is specific to corolla chromoplasts and is accumulated in parallel with flower development up to anthesis. Although its pattern of regulation by environmental and developmental factors is identical with that of CHRC, the fact that its level in chromoplasts is very low as compared with, for example, that of the corolla carotenoid-associated CHRC protein makes it almost impossible to prove its association with carotenoids via the isolation of pigmented complexes. Nevertheless, since under all solubilization conditions leading to the resolution of the pigment-protein complex CHRD always co-migrated with the complex, its function, like that of CHRC, may be to aid in carotenoid sequestration within tubulous chromoplasts. Because the availability of pigment molecules may strongly affect apoprotein stability (Apel and Kloppstech, 1980; Bennett, 1981; Mullet et al., 1990), it would be intriguing to detail the effects of the developmental and environmental signals described here on the level of carotenoids and on the rates of CHRD synthesis versus turnover. Characterization of the gene(s) coding for CHRD and reconstitution studies should lead to a final clarification of this protein's function and the mechanisms regulating its accumulation in chromoplasts.

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