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Chromoplast Biogenesis in *Cucumis sativus* Corollas[1]

Rapid Effect of Gibberellin A_3 on the Accumulation of a Chromoplast-Specific Carotenoid-Associated Protein

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The development of cucumber (*Cucumis sativus* L.) corollas is accompanied by the accumulation of chromoplasts. In mature corollas, chromoplasts, but no chloroplasts, were detected by electron microscopy. Chlorophyll was also undetectable in corollas at anthesis. The contents of carotenoids and a carotenoid-associated, chromoplast-specific, 35-kD protein in corollas increased in parallel with flower development, peaking concomitantly at anthesis. The involvement of phytohormones and light in the regulation of their expression was studied. When gibberellin A_3 (GA_3) was added to an in vitro bud culture system, accumulation of both carotenoids and the 35-kD protein was markedly enhanced. The specific up-regulation of the 35-kD protein was very rapid: after only 2 h of culture, increased levels were detected in GA_3-treated versus untreated corollas. During this period, corolla fresh weight and total protein and carotenoid contents remained unchanged. Inclusion of abscisic acid in the culture medium counteracted the effect of GA_3. Accumulation of the 35-kD protein was also enhanced when flower buds on plants were sprayed with GA_3 or etiolated.

Mature corollas and fruits of many higher plants contain chromoplasts, which arise from proplastids or chloroplasts. Much information has been accumulated on the molecular anatomy of chloroplasts, including pigment organization in the thylakoids, and information on the molecular mechanisms regulating their biogenesis. A large number of photosynthesis-related genes of both nuclear and plastid origin have been characterized (Mullet, 1988; Gruissem, 1989; Marder and Barber, 1989; Thornber et al., 1991). The mode of light-controlled expression of these genes and that of chloroplast biogenesis have been detailed. Some progress has also been made toward understanding the effect of phytohormones on the expression of apoproteins and on chloroplast development (Silverthorne and Tobin, 1987; Mullet, 1988; Chory, 1991). However, much less attention has been directed toward understanding the developmentally controlled differentiation of proplastids and chloroplasts into chromoplasts. The limited information that is available from studies performed with fruits indicates the involvement of phytohormones and light in regulating chromoplast biogenesis. Treatment of green fruits with ethylene led to the accumulation of chromoplasts, whereas GA or light treatments delayed the chloroplast-chromoplast conversion and even enhanced regreening of colored fruits (Coggins and Lewis, 1962; Thomson et al., 1967; Devide and Ljubesic, 1974; Goldschmidt, 1988; Boyer, 1989; Pfander, 1992).

The development of chromoplasts is accompanied by the appearance of a new set of proteins and by the accumulation of carotenoids, concomitant with the disappearance of chloroplast-specific components (Hansmann and Sitte, 1982; Bathgate et al., 1985; Newman et al., 1989; Milicua et al., 1991). Almost nothing is known about the mechanism(s) regulating expression of the chromoplast-specific proteins, although some progress has been made in detailing the carotenoid biosynthesis pathway in higher plants (Pfander, 1992; Giuliano et al., 1993). To date, only two carotenoid-associated chromoplast-specific proteins have been isolated and immunologically characterized in higher plants: a 58-kD protein from pepper (*Capsicum annuum*) fruits (Newman et al., 1989; Cervantes-Cervantes et al., 1990) and a 35-kD protein (termed CHRC hereafter) from cucumber (*Cucumis sativus*) corollas (Smirra et al., 1993). These proteins were shown to peak in mature fruits and flowers, respectively.

In the present report, we characterize chromoplast development in cucumber corollas and study the involvement of phytohormones and light in regulating the expression of the CHRC protein and accumulation of carotenoids. Using an in vitro bud culture system resembling flower development in vivo with respect to patterns of CHRC and carotenoid accumulation (Smirra et al., 1993), the level of CHRC is shown to be very rapidly enhanced following the inclusion of GA_3 in the medium.

MATERIALS AND METHODS

Plant Material

Cucumis sativus L. plants (cv Shimshon, obtained from Zeraim, Gedera, Israel) were grown under standard greenhouse conditions. When indicated, young green flower buds (approximately 5 mm in length; stage 1) were either heavily misted once a day with an aqueous solution of 0.8 mM GA_3

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Abbreviation: CHRC, chromoplast-specific protein of 35 kD.

or water (control), or enclosed in aluminum foil for specified periods of time (etiolation experiment).

In Vitro Culture

Stage 1 flower buds were collected, rinsed several times with sterile water, and placed in a culture vessel containing an aqueous solution of 150 mM Suc. When indicated, 0.4 mM GA_3 or 0.1 mM ABA was included in the culture media, or ethylene (up to 3 $\mu L\ L^{-1}$) was included in the vessel atmosphere. Buds were placed on a floating stand (a perforated microwell plate cover) inside the vessels so that only their bases were in contact with the culture medium, and they were cultured for the specified periods of time, as previously described (Smirra et al., 1993).

SDS-PAGE and Protein Transfer to Nitrocellulose Filters

Extraction of total proteins from corollas, SDS-PAGE, and western blotting using affinity-purified polyclonal antibodies against CHRC were performed as previously described (Smirra et al., 1993). ^{125}I-Protein A was used for antibody-antigen decoration. Autoradiogram quantification was carried out by scanning appropriately exposed films in a densitometer (Molecular Dynamics, Sunnyvale, CA) and by scintillation counting of excised radioactive bands. Each experiment was repeated at least three times with two amounts of protein (10 and 25 μg/lane) for each sample. Exposure times differed for the different blots.

EM

Corolla and leaf segments (2 mm × 3 mm) were fixed for 4 h in 4% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7, at 0°C. The segments were then washed three times in the buffer and postfixed in 2% (w/v) OsO_4 for 4 h at 4°C. The tissue was dehydrated and embedded in Spurr's resin and thin sections were prepared for EM observation in a JEOL JEM 100 CX, as previously described (Vainstein and Sharon, 1993).

Analytical Methods

To determine dry weight, freshly collected corollas were dried for 48 h at 60°C. Protein content was determined according to the method of Lowry et al. (1951). Chl and carotenoid contents were determined as described by Lichtenthaler (1987).

RESULTS

Chromoplast Biogenesis during Cucumber Flower Development

Figure 1 illustrates developmental stages of cucumber flowers, from a small bud with the corolla just beginning to appear (stage 1) to corollas 24 h after anthesis (stage 5). Stage 1 corollas were green, whereas fully developed corollas at anthesis (stage 4) were yellow. Stages 1, 2, and 3 occurred 120, 72, and 24 h before anthesis, respectively.

Dry weight, protein, and carotenoid contents of corollas increased to stage 4 of flower development (Table I). Whereas the levels of the first two parameters increased about 10-fold, carotenoid content increased about 30-fold from stages 1 to 4. Similar to the latter was the increase in content of the chromoplast-specific, carotenoid-associated protein, CHRC, in the corollas at these stages (Table I). The increase in carotenoid content of corollas from stages 3 to 4 was smaller (about 2.5-fold) than that in fresh weight (about 4-fold). During this time, total protein content increased by only approximately 20%. Corolla Chl content increased to stage 2, then decreased markedly to undetectable levels in stage 4 corollas (Table I).

At stage 4 of flower development, chromoplasts but no chloroplasts were detected by EM in corollas. Young corollas, on the other hand, contained chloroplasts that were structurally similar to those in leaves (Fig. 2).

Effect of GA_3 on the Accumulation of CHRC and Carotenoids in Corollas Cultured in Vitro

GA_3 was added to the in vitro culture medium, or ethylene was added to the vessel atmosphere, to study their effects on the levels of carotenoids and CHRC in corollas. Neither level was affected by ethylene at concentrations of up to 3 $\mu L\ L^{-1}$ (data not shown). In contrast, inclusion of GA_3 resulted in the enhanced accumulation of both carotenoids and CHRC (Figs. 3 and 4, respectively). After 2 d of culture in the presence of GA_3, the carotenoid content of corollas was twice the carotenoid content of those cultured in the absence of GA_3. When calculated per unit fresh weight, the increase in carotenoid level in GA_3-treated versus untreated corollas was also apparent on the second day of culture, with no detectable influence of GA_3 on carotenoid content during the first 6 h of culture. In contrast, the level of CHRC in corollas was very rapidly affected following the inclusion of GA_3 in the culture medium (Fig. 4). After only 2 h, corollas grown in the presence of GA_3 had accumulated, per unit protein, 1.8 ± 0.2 times higher levels of CHRC as compared with those grown without GA_3. After 6 h of culture (also based on autoradiogram quantitation), the level of CHRC was 3.2 ± 0.3 times higher in treated versus untreated corollas. No change in corolla fresh weight or total protein content could be detected during the first 6 h of in vitro culture, regardless of treatment.

To test whether ABA, known to be antagonistic to GA_3 in

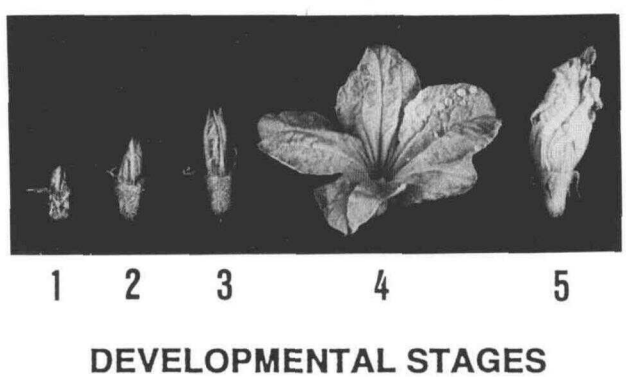

Figure 1. Developmental stages (1–5) of cucumber flowers.

Table I. *Weight, protein, and pigment contents of corollas at various stages of development*

Means of four replications ± SE. Relative CHRC levels per corolla expressed in arbitrary units (a.u.) were determined as described in "Materials and Methods." ND, Not detectable.

Sample	Fresh Weight	Dry Weight	Protein	Chl	Carotenoids	CHRC
	mg/corolla	*mg/corolla*	*μg/corolla*	*μg/corolla*	*μg/corolla*	*a.u.*
Corolla						
Stage 1	5.8 ± 0.6	1.1 ± 0.1	62.5 ± 7.1	1.3 ± 0.1	0.8 ± 0.1	10 ± 2
Stage 2	15.3 ± 1.4	2.1 ± 0.2	196.6 ± 18.2	2.6 ± 0.2	3.2 ± 0.4	70 ± 9
Stage 3	33.7 ± 3.5	3.6 ± 0.4	453.5 ± 30.5	1.8 ± 0.1	9.7 ± 1.0	250 ± 22
Stage 4	139.6 ± 14.2	10.2 ± 0.9	536.5 ± 40.2	ND	24.2 ± 2.3	310 ± 25
Stage 5	96.8 ± 9.5	6.1 ± 0.5	350.0 ± 34.3	ND	11.4 ± 0.9	8 ± 1

several systems (Pharis and King, 1985; Huttly and Baulcombe, 1989; Shi et al., 1992), would also counteract the GA_3 effect observed here, it was added to the culture medium. CHRC level in corollas cultured in the presence of ABA was not enhanced following inclusion of GA_3 and remained lower than that in control corollas cultured in the absence of ABA (Fig. 4B).

Effects of GA_3 and Etiolation on the Accumulation of CHRC in Corollas in Vivo

The effect of GA_3 on CHRC accumulation was monitored in vivo (Fig. 5). The level of CHRC in corollas of buds sprayed with GA_3 was 2.1 ± 0.2 times higher than in those sprayed with water, per unit protein. To test the effect of light, the

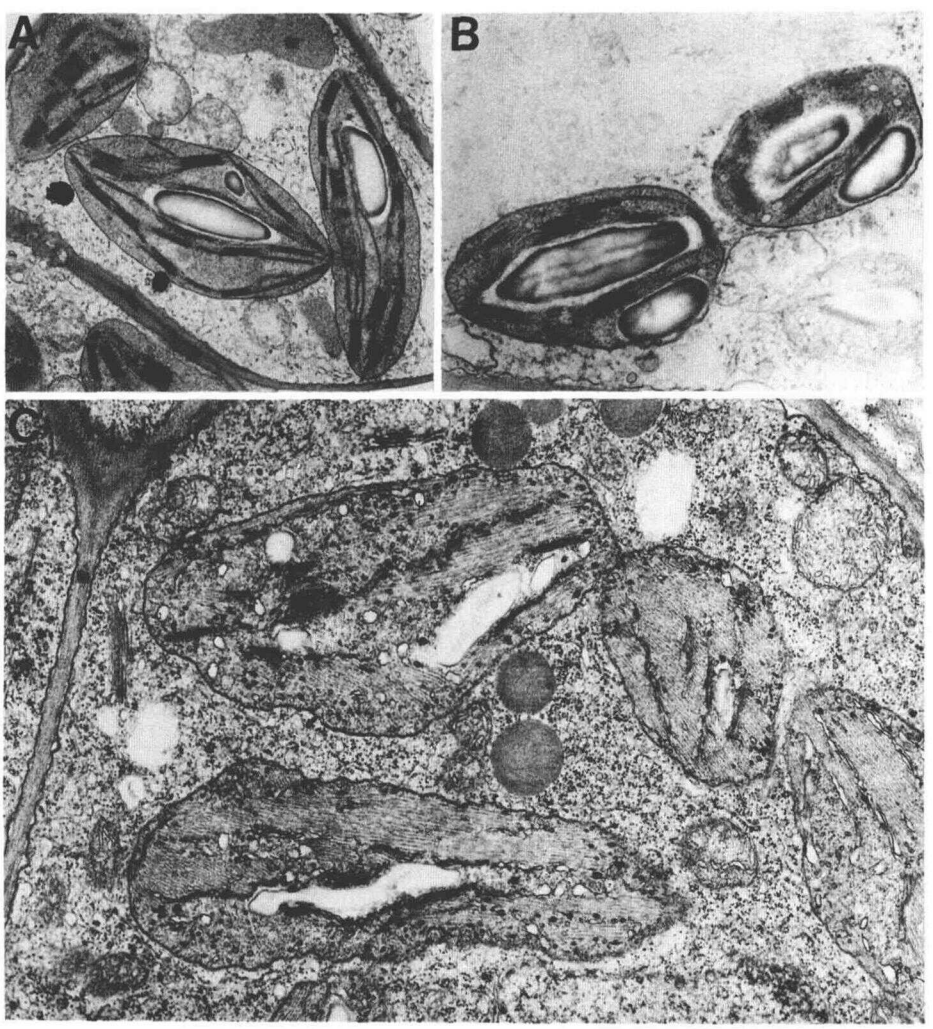

Figure 2. Electron micrographs of thin sections of corollas and young leaves. A, Leaf; B and C, corollas at stages 1 and 4, respectively. Magnification: A, ×9,200; B, ×11,400; C, ×34,400.

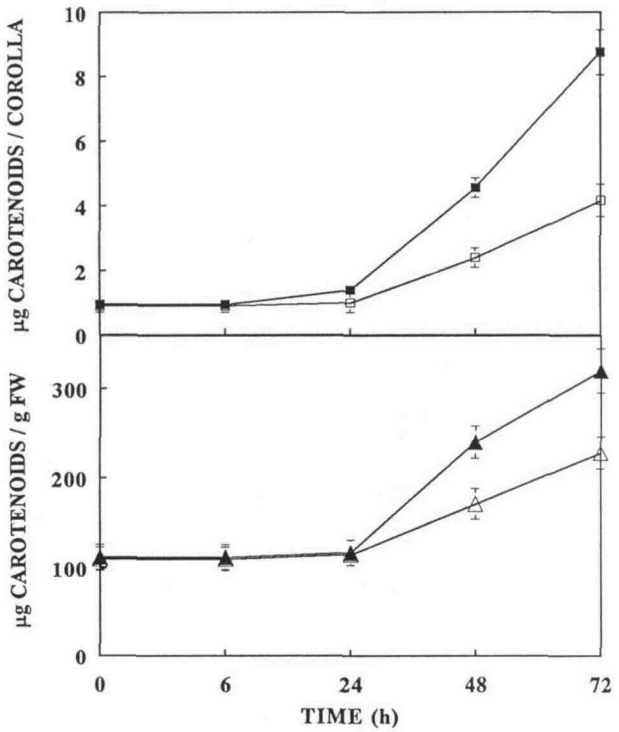

Figure 3. Effect of GA_3 on the carotenoid content of corollas during flower development in vitro. Young flower buds were cultured for the indicated periods of time in Suc-containing media with GA_3 (solid symbols) or without GA_3 (open symbols). Carotenoid levels ± SE were determined as described in "Materials and Methods."

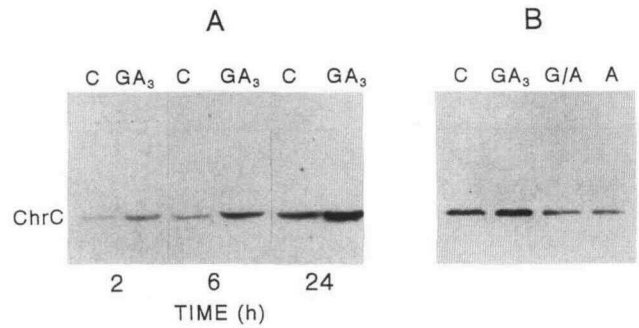

Figure 4. Effect of GA_3 on the CHRC content of in vitro-cultured corollas. Young flower buds were cultured in a Suc-containing medium with (GA_3) or without (C) GA_3. At the indicated periods of time, corollas were withdrawn and total protein content (25 µg) was analyzed by western blotting using antibodies against CHRC (A). B, Western blot analysis (15 µg total protein/lane) of CHRC levels in corollas cultured for 24 h in the presence of GA_3 (GA_3), ABA (A), GA_3 and ABA (G/A), or without phytohormones (C).

level of CHRC in etiolated buds was analyzed (Fig. 5). The CHRC level in corollas of etiolated buds was 1.9 ± 0.2 times higher than that in control flowers, per unit protein. Carotenoid accumulation was also enhanced in both GA_3-treated and etiolated corollas (data not shown).

DISCUSSION

Maturation of *C. sativus* corollas is accompanied by the accumulation of chromoplasts and the disappearance of chloroplasts. At anthesis, only plastids with structures characteristic of chromoplasts could be found in corollas (Fig. 2, and Smith and Butler, 1971). Mature corollas contained high levels of carotenoids, whereas Chl was undetectable. Carotenoid content of corollas, as well as that of the carotenoid-associated protein CHRC, increased up to anthesis (stage 4), whereas when calculated per unit fresh weight, both peaked at an earlier stage of flower development (Table I, and Vainstein et al., 1992). A recent report has shown that transcript levels of genes coding for phytoene synthase and desaturase, as well as carotenoid levels, reach about two-thirds of their maximal levels in tomato corollas prior to anthesis (Giuliano et al., 1993). These latter results also suggest that unlike corolla growth, processes related to chromoplast buildup peak prior to anthesis.

Light, an environmental signal, induces the synthesis of chloroplast-specific apoproteins. On the other hand, the absence of light has been suggested to promote chromoplastogenesis (Thomson et al., 1967; Devide and Ljubesic, 1974; Goldschmidt, 1988; Boyer, 1989; Pfander, 1992). Carotenoids, unlike Chl, are also synthesized in the dark. Since the carotenoid-associated, chromoplast-specific protein CHRC was up-regulated in the dark (Fig. 5), an increase in the available carotenoid binding sites may be suggested as part of the mechanism leading to carotenoid accumulation in the dark. The observation that carotenoid content of corollas increases during etiolation (Vainstein et al., 1992) supports this suggestion.

Ethylene, a growth regulator often associated with fruit ripening and flower senescence (Halevy and Mayak, 1981; Borochov and Woodson, 1989), has also been shown to promote chromoplastogenesis in fruits (Goldschmidt, 1988). However, inclusion of ethylene in our in vitro bud culture system did not affect expression of either the carotenoids or CHRC. It is worth noting that the in vitro system presented in this study closely resembles flower development in vivo. After 3 d in culture, fresh weight, carotenoid, and CHRC contents of corollas were very similar to those accumulated in corollas of intact buds at stage 2 (Table I, Fig. 1, and Smirra

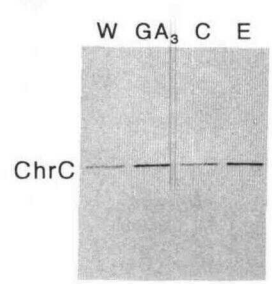

Figure 5. Effect of GA_3 or etiolation on CHRC levels in corollas of intact plants. Flower buds were sprayed with water (W), GA_3 (GA_3), or etiolated (E). Plants were then grown for 24 h and CHRC levels were analyzed by western blotting using 10 µg total protein/lane. C, Control (nonetiolated) plants. For details see "Materials and Methods."

et al., 1993). After 5 d of culture, corollas were very similar to those of intact buds at stage 3 (Vainstein et al., 1992). We could not assess flower development in vitro after longer time intervals since flowers were obtained from the greenhouse, and aseptic conditions could not be implemented.

In contrast to ethylene, levels of carotenoids and CHRC were enhanced following treatment of buds with GA_3. This up-regulation of CHRC was counteracted by ABA, similar to what has been reported in several previously described GA_3-controlled gene expression systems (Pharis and King, 1985; Huttly and Baulcombe, 1989; Shi et al., 1992). Involvement of GA in chromoplastogenesis has been previously described in fruits. In that system, however, an opposite trend was observed: GA delayed the conversion from chloroplasts to chromoplasts, and even induced the conversion from chromoplasts to chloroplasts (Coggins and Lewis, 1962; Goldschmidt, 1988; Pfander, 1992). GA_3 also up-regulates anthocyanin accumulation in petunia corollas (Weiss and Halevy, 1989). Thus, the same pigmentation pathway, i.e. that of chromoplast accumulation, can be affected by GA_3 in opposite ways in different chromoplast-containing tissues. In floral tissue GA_3 affects corolla coloration similarly, irrespective of whether pigmentation is due to anthocyanin or chromoplast accumulation.

The role of GA_3 in the regulation of gene expression has probably been most extensively studied in the aleurone layer of germinating cereal seeds (Ou-Lee et al., 1988). Regulation of chalcone synthase gene transcription in petunia corollas by GA_3 has also been reported (Weiss et al., 1992). In both systems, there was a relatively long lag time between GA_3 application and the accumulation of gene products, and the involvement of intermediate factors such as *trans*-regulatory proteins was suggested. A much shorter lag time, as in some other systems (Chory et al., 1987; Shi et al., 1992), was observed in vitro for the CHRC protein of corolla chromoplasts. Specific up-regulation of CHRC in corollas, following the inclusion of GA_3 in the culture medium, was already detectable after 2 h (Fig. 4). During this period, there were no detectable changes in corolla fresh weight or protein content. The level of chloroplast-specific, major light-harvesting Chl *a*/*b* protein, studied for comparison, was also not affected by GA_3 (data not shown).

Cloning the gene for CHRC may enable an elucidation of the mechanism(s) involved in GA_3-enhanced accumulation of this chromoplast-specific, carotenoid-associated protein in corollas. This would greatly assist in detailing the mechanisms involved in chromoplast biogenesis and carotenoid accumulation, and would enable an in-depth comparison of the transduction pathways by which GA_3 regulates various processes.

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LITERATURE CITED

Bathgate B, Purton ME, Grierson D, Goodenough PW (1985) Plastid changes during the conversion of chloroplasts to chromoplasts in ripening tomatoes. Planta **165:** 197–204

Borochov A, Woodson WR (1989) Physiology and biochemistry of flower petal senescence. Hortic Rev **11:** 15–43

Boyer CD (1989) Genetic control of chromoplast formation during fruit development of *Cucurbita pepo* L. *In* CD Boyer, JC Shannon, RC Harison, eds, Physiology, Biochemistry and Genetics of Non-green Plastids. Proceedings of the 4th Annual Penn State Symposium in Plant Physiology. American Society of Plant Physiologists, Rockville, MD, pp 241–252

Cervantes-Cervantes M, Hadjeb N, Newman LA, Price CA (1990) ChrA is a carotenoid-binding protein in chromoplasts of *Capsicum annuum*. Plant Physiol **93:** 1241–1243

Chory J (1991) Light signals in leaf and chloroplast development: photoreceptors and downstream responses in search of a transduction pathway. New Biol **3:** 538–548

Chory J, Voytas DF, Olszewski NE, Ausubel FM (1987) Gibberellin-induced changes in the populations of translatable mRNAs and accumulated polypeptides in dwarfs of maize and pea. Plant Physiol **83:** 15–23

Coggins CW, Lewis LN (1962) Regreening of Valencia orange as influenced by potassium gibberellate. Plant Physiol **37:** 625–627

Devide Z, Ljubesic N (1974) The reversion of chromoplasts to chloroplasts in pumpkin fruits. Z Pflanzenphysiol **73:** 296–306

Giuliano G, Bartley GE, Scolnic PA (1993) Regulation of carotenoid biosynthesis during tomato development. Plant Cell **5:** 379–387

Goldschmidt EE (1988) Regulatory aspects of chloro-chromoplast interconversions in senescing *Citrus* fruit peel. Isr J Bot **37:** 23–130

Gruissem W (1989) Chloroplast gene expression: how plants turn their plastids on. Cell **56:** 161–170

Halevy AH, Mayak S (1981) Senescence and postharvest physiology of cut flowers. Hortic Rev **3:** 59–143

Hansmann P, Sitte P (1982) Composition and molecular structure of chromoplast globules of *Viola tricolor*. Plant Cell Rep **1:** 111–114

Huttly AK, Baulcombe DC (1989) A wheat α-Amy2 promoter is regulated by gibberellin in transformed oat aleurone protoplasts. EMBO J **8:** 1907–1913

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol **148:** 350–382

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Marder JB, Barber J (1989) The molecular anatomy and function of thylakoid proteins. Plant Cell Environ **12:** 595–614

Milicua JCG, Juarros JL, De Las Rivas J, Ibbarondo J, Gomez R (1991) Isolation of a yellow carotenoprotein from carrot. Phytochemistry **30:** 1535–1537

Mullet JE (1988) Chloroplast development and gene expression. Annu Rev Plant Physiol Plant Mol Biol **39:** 475–502

Newman LA, Hadjeb N, Price CA (1989) Synthesis of two chromoplast-specific proteins during fruit development in *Capsicum annuum*. Plant Physiol **91:** 455–458

Ou-Lee TM, Tugeon R, Wu R (1988) Interaction of gibberellin-induced factor with the upstream region of an α-amylase gene in rice aleurone tissue. Proc Natl Acad Sci USA **85:** 6366–6369

Pfander H (1992) Carotenoids: an overview. Methods Enzymol **213:** 3–13

Pharis RP, King RW (1985) Gibberellins and reproductive development in seed plants. Annu Rev Plant Physiol **36:** 517–568

Shi L, Gast RT, Gopalraj M, Olszewski NE (1992) Characterization of a shoot-specific, GA_3- and ABA-regulated gene from tomato. Plant J **2:** 153–159

Silverthorne J, Tobin EM (1987) Phytochrome regulation of nuclear gene expression. BioEssays **71:** 18–22

Smirra I, Halevy AH, Vainstein A (1993) Isolation and characterization of a chromoplast-specific carotenoid-associated protein from *Cucumis sativus* corollas. Plant Physiol **102:** 491–496

Smith M, Butler RD (1971) Ultrastructural aspects of petal development in *Cucumis sativus* with particular reference to the chromoplasts. Protoplasma **73:** 1–13

Thomson WW, Lewis LN, Coggins CW (1967) The reversion of chromoplasts to chloroplasts in Valencia oranges. Cytologia **32:** 117–124

Thornber JP, Morishige DJ, Anandan S, Peter GF (1991) Chlorophyll-carotenoid proteins of higher plant thylakoids. *In* H Scheer, ed, Chlorophylls. CRC Press, Boca Raton, FL, pp 549–585

Vainstein A, Halevy AH, Smirra I (1992) Chloroplast-chromoplast conversion during development of *Cucumis sativus* flowers. *In* Proceedings International Workshop: Molecular Control of Flower Development and Plant Reproduction. Amsterdam, The Netherlands, p 73

Vainstein A, Sharon R (1993) Biogenesis of petunia and carnation corolla chloroplasts: changes in the abundance of nuclear and plastid-encoded photosynthetic gene products during flower development. Physiol Plant **89:** 192–198

Weiss D, Blokland RV, Kooter JH, Mol JNM, van Tunen AJV (1992) Gibberellic acid regulates chalcone synthase gene transcription in the corolla of *Petunia hybrida*. Plant Physiol **98:** 191–197

Weiss D, Halevy AH (1989) Stamens and gibberellin in the regulation of corolla pigmentation and growth in *Petunia hybrida*. Planta **179:** 89–96