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Genetic control of plastid carotenoids and transformation in the skin of *Cucurbita pepo* L. fruit*

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Summary. The influence of allelic state of gene *B* on skin pigmentation in two cultivars of *Cucurbita pepo* L. has been studied. Total carotenoids were lower at early stages of fruit development in cultivar (cv.) 'Early Prolific' (EP) *BB YY* fruit skin, than in EP B^+B^+ *YY* fruit skin, but no differences were observed in total skin carotenoids twenty days after anthesis. Total carotenoids were lower in cv. 'Fordhook Zucchini' (FZ) *BB yy* fruit skin, than in FZ B^+B^+ *yy* fruit skin at all developmental stages from anthesis to maturity. Both green and yellow tissues contained typical foliar carotenoids. The carotenoids from yellow fruit skin of both EP genotypes and of FZ *BB* were characterized by a low carotene : xanthophyll ratio, with a high proportion of the xanthophylls esterified to fatty acids. The xanthophylls of the yellow tissues were esterified with 12 : 0, 14 : 0, 16 : 0 fatty acids. The carotenoids from the green fruit skin of FZ B^+B^+ had a higher percentage of carotenes (primarily β-carotene) and a lower percentage of esterified xanthophylls. Spectral shapes of carotenoid fractions from all yellow tissues were similar and distinguishable from those of green FZ B^+B^+ tissue. The results of these studies are discussed in terms of the genetic control of plastid transformation in *Cucurbita pepo* L.

Key words: Chloroplast – Chromoplast – Squash – *Cucurbita*-transformation

Introduction

Cucurbita pepo L. fruits have a wide variety of colors and developmental pigmentation patterns (Shifriss 1949). For example, the ovary of the cultivar EP B^+B^+ *YY* is green initially and begins to turn yellow under the influence of the *Y* allele near the time of anthesis (Schaffer and Boyer 1984; Shifriss 1981). FZ B^+B^+ *yy* ovaries also are green prior to anthesis, but the fruit remains green throughout development due to *yy* (Sinnott and Durham 1922). Near isogenic lines for the *B* allele of both cultivars are non-green prior to anthesis, and apparently do not develop any chlorophyll in the colored rind. During development, EP *BB YY* fruits change from cream-yellow to yellow while FZ *BB yy* fruits become more intensely pigmented changing from yellow to orange in color (Schaffer and Boyer 1984).

The yellow color of the ovaries from *B* plants has been described as precociously pigmented (Shifriss 1955). The origin of *B* is the bicolor ornamental gourd *C. pepo* var. 'ovifera' (Shifriss 1981). Ljubesic (1970, 1972) and Devide (1970) studied the ultrastructural development of plastids of the bicolor ornamental gourds and found that the precociously yellow portions have chromoplasts which develop from proplastids. In varieties where the green portion later turns orange, the chromoplasts are derived from chloroplasts. Grilli (1965a, b) studied the development of chromoplasts of *C. pepo* cultivar (cv.) 'Small Sugar', a small pumpkin, that is green until approximately twenty days past anthesis and then turns orange. She found that the chromoplasts of the heavily pigmented outer pericarp were derived from chloroplasts and proplastids, but flesh chromoplasts were derived from amyloplasts. She also reported that xanthophylls predominated in the pigmented outer pericarp, while carotenes were the major pigments of the flesh chromoplasts.

Chromoplasts can differ greatly in their pigment composition (Goodwin 1976). Fruits may be characterized by chromoplast specific carotenoids, i.e. lycopene of tomatoes and capsorubin and capsanthin of red peppers. In these cases, the

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chloroplast-to-chromoplast transformation generally is accompanied by rapid carotenoid synthesis. On the other hand, some fruits, i.e. yellow peppers (Davies et al. 1970), and Cox's Orange Pippin apple (Knee 1972) undergo chloroplast-to-chromoplast transformation but maintain the foliar carotenoids, β-carotene, lutein, violaxanthin and neoxanthin. Fruit chloroplasts from immature green tomatoes (Rabinowitch 1975), peppers (Davies et al. 1970), and apples (Knee 1972), contain typical foliar chloroplast carotenoids in which approximately 20–25% of the pigments are non-hydroxylated hydrocarbons. Chromoplasts which contain chloroplast carotenoids often are characterized by a low ratio of carotenes to xanthophylls. For example, ripe yellow peppers contain 1% hydrocarbons compared to 24% in green peppers (Davies et al. 1970). Similar observation have been reported in apples (Knee 1972), oranges (Eilati 1972), and senescing autumn leaves (Grob and Eichenberger 1962). In addition to changes in pigment composition, fatty acid esterification of xanthophylls occurs during chloroplast to chromoplast transformations (Davies et al. 1970; Knee 1972; Eilati et al. 1972; Philip 1973; Booth 1964). Egger (1968) studied the fatty acids of *Helianthus anuum* L. flowers derived from esterified xanthophylls and found stearic, palmitic, myristic, lauric and acetic acid esterified to lutein, violaxanthin, cryptoxanthin and taraxanthin.

Previously, we have demonstrated that the *B* gene does not affect the final fruit color of *C. pepo* cvs. which are homozygous *YY* (Schaffer and Boyer 1983a; Schaffer and Boyer 1984). However, Shifriss (1981) reported that *B* genotypes produced fruit with higher total carotenoid content. The purpose of this study was to characterize carotenoid development and content of *C. pepo* fruit skin in order to determine the effect of genes *B* and *Y* on fruit pigmentation using the following four genotypes: EP *BB YY*, EP B^+B^+ *YY*, FZ *BB yy* and FZ B^+B^+ *yy*.

Materials and methods

Plant material

Derivation of plant material and isogenic lines has been described previously (Schaffer and Boyer 1984; Shifriss 1982). After germination in a greenhouse, seedlings were transplanted to the field. Open-pollinated ovaries were tagged on the day of anthesis and fruit were harvested at various developmental stages from anthesis to 60 days past anthesis.

Pigment analysis

Fruit skin was sampled by scraping the skin to the depth of either the lignified rind, when present, or the petal and sepal vascular bundles. Since the pepo fruit is an inferior ovary, this insured that the tissue sampled consisted of modified receptacle tissue (Judson 1929). Samples, 0.5 to 5.0 g, were taken and stored at −5 °C under N_2. An additional sample from each fruit was weighed, oven dried, and data recorded for percent dry weight. Data also was taken on the area of fruit skin sampled, except for anthesis-stage fruit.

Three to six samples of each genotype at each developmental stage studied were individually extracted in subdued light as follows. The frozen tissue was homogenized in 75 ml cold acetone for 15 min. The homogenate was covered with N_2 and extracted for approximately 16 h at 5 °C in the dark to completely solubilize the pigments. The acetone extract was filtered and the pigments extracted into 50 ml petroleum ether by the addition of NaCl saturated H_2O. The aqueous hypophase was repeatedly extracted with petroleum ether until clear and colorless. The bulked petroleum ether fraction were washed with H_2O and dried over NaCl. Ten percent of the sample was used for total carotenoid analysis while the remaining 90% of the sample was used for analysis of individual carotenoids.

Total carotenoids

The petroleum ether was evaporated under N_2 and the pigments redissolved in ethanol to which 60% aq. KOH (w/v) was added to a final concentration of approximately 6% KOH. After saponification at 56 °C for 45 min in the dark, the pigments were partitioned into diethyl ether. The ether was repeatedly washed with H_2O to remove the alkali and dried over NaCl. The solvent was evaporated under N_2 and the pigments redissolved in a convenient volume of petroleum ether and diluted as necessary.

Absorption spectra in the range of 500–400 nm were recorded. Total carotenoids were calculated using the maximum absorbance of the central peak (445–438 nm) and the specific extinction coefficient of 2,500 (Davies 1976), and expressed as μg β-carotene equivalents per, gram fresh weight, gram dry weight or cm^2.

Carotenoid fractions

The petroleum ether was reduced in vacuo at 35 °C to approximately 1 ml. Separation into three fractions (designated A, B, and C) was carried out according to a modification of the procedure of Eilati et al. (1972). The pigments were loaded onto a 16 cm × 1.2 cm column of Adsorption Alumina (Fisher Scientific Co., Springfield, NJ) Na_2SO_4 (1/1, w/w). Carotenoids without free hydroxyl groups (carotenes plus esterified xanthophylls) were eluted with 4% acetone/petroleum ether (v/v) until the eluate appeared clear. Carotenoids with one or more free hydroxyl groups were eluted with 20% ethanol/petroleum ether (v/v). Both column eluates were evaporated to near dryness under N_2, and redissolved in approximately 3 ml ethanol and 0.3 ml aq. 60% KOH and saponified. Each eluate was then rechromatographed on identical alumina columns, with the same solvents used previously. The acetone/petroleum ether eluate yielded both non-hydroxylated (fraction A) and hydroxylated pigments (fraction B), while the ethanol/petroleum ether eluate yielded only hydroxylated pigments (fraction C).

Each of the three fractions were brought up to convenient volumes with petroleum ether. Aliquots were diluted as necessary, and absorption spectra in the range of 500–400 nm of each fraction were recorded. Carotenoids in each fraction were determined spectrophotometrically as above and was expressed as percent in the sum of the three fractions.

The three fractions, and the total carotenoid extracts, were chromatographed in the dark on silica TLC plates (200 μm Whatman LK5F) using 30% acetone/petroleum ether (v/v) according to a modification of Gross (1980). Individual bands were eluted with ethyl ether, and occasionally rechromatographed on MgO : Hyflo Super Cel TLC plates (200 μm) to separate α-ionone and β-ionone isomers (Britton and Goodwin 1971).

Identification of individual pigments was made by cochromatography with standards (α-carotene and β-carotene, Sigma Chemical Co.; lutein, violaxanthin, and neoxanthin obtained from spinach leaves; lycopene from tomatoes; cryptoxanthin from *Physalis alkegengi*), using color reactions on TLC plates after exposure to HCl vapors, and absorption spec-

tra and chemical tests according to the methods described by Davies (1976).

Chemical identification tests

Pigments were characterized further by several chemical tests. The presence of 5,6-epoxy groups was demonstrated by the change in spectra caused by the addition of a few drops 0.05 N HCl/ethanol (Jungalwala and Cama 1962). Absorption spectra after transformation showed sharper absorption peaks. Cis-trans isomers were determined by hypsochromic shifts caused by iodine isomerization (Davies 1976; Jungalwala and Cama 1962). The presence of free hydroxyl groups was tested by increased Rf values on silica gel after acetylation (Davies 1976).

Mild saponification studies

Pigments were extracted as above and transferred to approximately 50 ml petroleum ether. The pigments were partitioned between petroleum ether and aqueous 85% MeOH 20% KOH (v/w), shaken vigorously for 3 min and allowed to sit in the dark for approximately 1 h. The hypophase, containing chlorophyll breakdown products and free xanthophylls, was discarded and the epiphase, containing carotenes and esterified xanthophylls was reextracted with aqueous 85% MeOH until the aqueous phase was clear, washed to remove the MeOH and dried over NaCl (Tomes 1963). The petroleum ether was evaporated under N_2 to a convenient volume, and the carotenoids characterized and quantified spectrophotometrically. A 100 µg aliquot was used for magnesia column chromatography (Jungalwala and Cama 1962).

Pigments were separated on a 10 × 1 cm MgO: Hyflo Super Cel (1 : 1 w/w) column and eluted with a 2%–15% acetone/petroleum ether linear gradient. Five ml fractions were collected and absorbance measured at 450 nm. Individual fractions, after removal of acetone, were characterized spectrophotometrically by spectral shapes, absorption maxima and chemical tests as described above.

Fatty acid methyl ester preparation

Methods used in the study of fatty acids esterified to xanthophylls were modified from Philip (1973) using fruit skin (11 g) from FZ *BB* 30 days past anthesis. The diester fraction was purfied by silica TLC using 6% acetone/petroleum ether as the mobile phase. The major band was scraped off, eluted with ethyl ether and rechromatographed on silica with 4% acetone/petroleum ether. The purified fraction was characterized by visible and UV spectra, tested for epoxide groups and cis-trans isomerization, and the carotenoid moiety was tentatively identified as lutein.

The pigment was transferred to ethanol and saponified as above. After cooling, the alkaline solution was acidified to pH 3.0 and the fatty acid, extracted into ether. After washing and drying with NaCl, the ether was removed under N_2 gas and the fatty acids were dissolved in 3 ml BF_3 in 12% methanol (Kodak), and heated at 70° for 2 min to methylate the fatty acids. The fatty acid methyl esters were transferred to ether and then to 0.5 ml ethyl acetate.

GC analysis

One µl of the fatty acid methyl ester solution was chromatographed on a silanized glass column, 1.8 m × 2.0 mm, packed with 10% DEGS on Chromasorb (Supelco), 80–100 mesh. Column temperature was maintained at 120 °C for 2 min, increased to 190 °C at 10 °C/min, and held at 190° for 5 min. N_2 was used as carrier gas at 30 ml/min, injection temperature was 220 °C, and organic compounds were detected by flame ionization at 300 °C. Standard fatty acids, 8 : 0, 10 : 0, 12 : 0, 14 : 0, 16 : 0, 18 : 0, 18 : 1, 18 : 2, 18 : 3, (carbon chain length: double bonds), were chromatographed under identical conditions, retention times recorded, and used for tentative identification.

Gas chromatography – mass spectrometry (GC-MS)

Samples in hexane were chromatographed on 50 mm × 0.2 mm fused silica capillary columns of Carbowax 20 M with a splitless injection of 2 µl. Operating conditions were 50 °C for 0.5 min, temperature increased at 8 °C/min for 14 min and then 5°/min to a final temperature of 210 °C. Mass range from 29–320 atomic mass units was scanned and spectra compared to those of the National Bureau of Standards Library.

Results

Total carotenoids and carotenoid fractions of fruit skin

Total carotenoid in fruit skin increased during the development of all genotypes studied. Skin from EP fruit contained less total carotenoids than skin from FZ fruits throughout development regardless of the allelic state of the *B* locus (compare Tables 1 and 2). In addition, the increases in carotenoids in fruit skin of EP genotypes were linear from 10 to 30 days after anthesis, while the increase in carotenoids of the fruit skin of FZ genotypes were biphasic, with a lag phase between 20 and 30 days after anthesis.

Table 1. Influence of *B* on total carotenoid concentration in Fordhook Zucchini (FZ) fruit skin

A Fresh weight basis (µg/g)

	Days				
Genotype	Anthesis	10	20	30	60
B^+B^+yy	113.2a[a]	228.9a	1,449.2a	1,567.9a	3,526.0a
$BByy$	51.0b	145.3a	1,028.1b	669.8b	1,486.4b

B Dry weight basis (µg/g)

	Days				
Genotype	Anthesis	10	20	30	60
B^+B^+yy	1,405a	2,403a	5,141a	4,405a	10,326a
$BByy$	582b	1,541a	4,268b	2,939b	5,873b

C Area basis (µg/cm²)

	Days				
Genotype	Anthesis	10	20	30	60
B^+B^+yy	ND[b]	10.15a	32.96a	35.50a	98.00a
$BByy$	ND	3.89b	16.28b	11.56b	38.27b

[a] Averages followed by different letters within a subheading and within a column, are different at the 0.05 level, using student's *t*-test

[b] ND = not determined

Table 2. Influence of *B* on total carotenoid concentration in Early Prolific (EP) fruit skin

A Fresh weight basis (μg/g)			
	Days after anthesis		
Genotype	10	20	30
B^+B^+ *YY*	24.5a[a]	272.8a	470.4a
BB YY	13.2b	243.3a	487.5a
B Dry weight basis (μg/g)			
	Days after anthesis		
Genotype	10	20	30
B^+B^+ *YY*	250.0a	881.4a	1,316.2a
BB YY	147.6b	1,079.7a	1,402.3a
C Area basis (μg/cm²)			
	Days after anthesis		
Genotype	10	20	30
B^+B^+ *YY*	1.37a	4.42a	6.69a
BB YY	0.87b	3.66a	6.33a

[a] Averages followed by different letters within a subheading and within a column, are different at the 0.05 level, using student's *t*-test

Table 3. Changes in the distribution of carotenoid fractions (percent of total) isolated from fruit skin during development

Days	Fraction	Genotype			
		FZ B^+B^+yy	FZ *BByy*	EP B^+B^+ *YY*	EP *BB YY*
Anthesis	C[a]	18.3	3.2	ND[d]	ND
	EX[b]	2.6	14.8	ND	ND
	X[c]	79.1	82.0	ND	ND
10	C	28.7	2.0	1.0	3.8
	EX	2.8	41.4	50.0	42.1
	X	68.5	56.5	48.1	54.1
20	C	23.9	4.4	3.8	3.5
	EX	12.1	29.1	43.6	30.4
	X	64.0	66.5	52.7	66.1
30	C	24.7	3.2	4.7	4.2
	EX	13.7	34.3	36.2	30.8
	X	61.6	62.5	59.1	65.0
60	C	16.6	6.6	ND	ND
	EX	15.0	23.7	ND	ND
	X	68.4	69.6	ND	ND

[a] C = carotenes
[b] EX = esterified xanthophylls
[c] X = free xanthophylls
[d] ND = not determined

Compared to FZ B^+B^+ *yy*, FZ *BB yy* fruit skin had lower levels of carotenoids at all developmental stages studied (Table 1). Although the differences in total carotenoids of skin of FZ B^+B^+ *yy* and FZ *BB yy* were large at 10 days after anthesis, they were not statistically significant due to high variability at this stage of development. In contrast, total carotenoids from skin of EP *BB YY* and EP B^+B^+ *YY* fruit were only different at 10 days after anthesis and were similar by 20 days after anthesis (Table 2).

The skin from the green fruit of FZ B^+B^+ *yy* had a typical foliar carotenoid pattern, with a large carotene (fraction A) to xanthophyll (fraction C) ratio of approximately 20%, and a relatively small percentage of esterified xanthophyll (fraction B), at both anthesis and 10 days after anthesis. By 20 days after anthesis, the esterified xanthophyll fraction increased and the ratio remained relatively unchanged thereafter (Table 3). In contrast, skin from all yellow fruits (EP B^+B^+ *YY*, EP *BB YY*, FZ *BB yy*) had a low carotene to xanthophyll ratio, and a high percentage of esterified xanthophylls as expected for chromoplast carotenoids. The only exception was FZ *BB yy* at anthesis which had a relatively low (14.8%) esterfied xanthophyll fraction. There were general similarities in pigment composition of the skin of fruit from all of the yellow lines, although skin from fruit of FZ *BB yy*, and EP *BB YY* had more similar percentages of esterified xanthophylls than EP *BB YY* and EP B^+B^+ *YY* (Fig. 1).

Interestingly, the free xanthophyll percentages of FZ B^+B^+ and FZ *BB* are very similar at all developmental stages studied, with the exception of 10 days after anthesis, regardless of the differences in carotenes and esterified xanthophylls. This suggests that there may be some upper limit to the level of free xanthophylls that a *C. pepo* skin plastid can maintain.

Spectral analysis of the saponified total carotenoid extracts substantiated the chromoplast-chloroplast distinction. Total carotenoids from fruit skin of all four lines, at all dates tested, had approximately the same

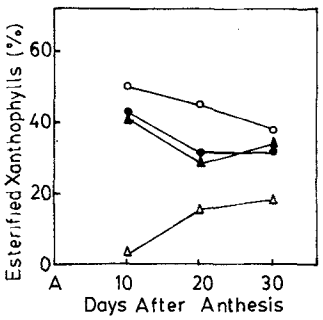

Fig. 1. Changes in percent esterified xanthophylls in the skin of fruit of different genotypes during development, ○ EP B^+B^+ *YY*; ● EP *BB YY*; △ FZ B^+B^+ *yy*; ▲ FZ *BB yy*

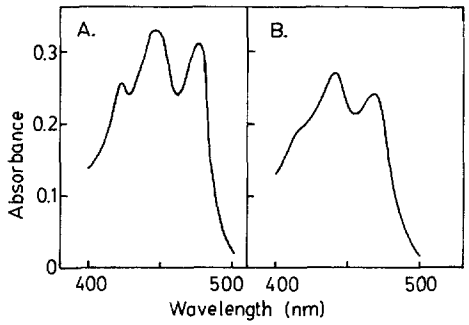

Fig. 2A, B. Absorption spectra of total saponified carotenoids isolated from the skin of Fordhook Zucchini fruit harvested at anthesis. **A** FZ *BB yy;* **B** FZ B^+B^+ *yy*

spectral peaks. However, carotenoids from all the developmental stages of FZ B^+B^+ *yy* had a spectral shape distinctly different from those of EP B^+B^+ *YY,* EP *BB YY* and FZ *BB yy* (Fig. 2). Peak I (in the 415–425 nm region) of the FZ B^+B^+ *yy* skin carotenoids consistently was a shoulder which always had an absorption lower than the 457 nm absorption, which is the valley between peaks II and III and serves as a baseline reference point (Fig. 2B). Absorption spectra of carotenoids from yellow fruit skins (Fig. 2A) always had a very sharp peak I with a higher absorbance than the 457 nm baseline. This difference most likely is due to the larger concentration of β-carotene in the green tissue (see "Pigment characterization").

Pigment characterization

The major pigments from skin of both green and yellow fruit were identified as the foliar carotenoids β-carotene, lutein, violaxanthin and neoxanthin. In addition, a major pigment with an Rf value intermediate between lutein and violaxanthin when chromatographed on silica gel was observed. This pigment appears to have two hydroxyl groups and a 5,6-epoxide group. Acid vapor treatment of the TLC plate turned the pigment light green, and acid treatment of the pigment produced a hypochromic shift of approximately 15 nm, indicating a mono-5,6-epoxide. The absorption peaks of this pigment in ethanol were 470 nm, 441 nm, 420 nm, suggesting taraxanthin, the α-ionone isomer of antheraxanthin (Booth 1964).

A study of the absorption spectra of the carotene, esterified xanthophyll, and free xanthophyll fractions from skin of yellow fruit (Fig. 3), showed distinguishable differences between the three fractions. The hydrocarbon fraction (Fig. 3C) has a typical β-carotene shape with a characteristically low III/II ratio and a low shoulder in the 425 nm region. Assuming that esterification should not change the absorption spectrum of xanthophylls, the differences between the free and esterified xanthophyll spectra require explanation. The spectrum of the free xanthophyll (Fig. 3A) fraction with a high peak I resembles the spectrum of neoxanthin (Bickoff et al. 1954), while the esterified xanthophyll (Fig. 3B) spectrum with a low peak I relative to the 457 nm peak resembles the spectrum of lutein. If neoxanthin was not completely esterified, the free xanthophyll fraction may contain partially esterified neoxanthin as well. Neoxanthin has 3 hydroxyl groups, two of which are 2° while the third is 3°. If the 3° hydroxyl group was not as readily esterified, neoxanthin might have been esterified at only the two 2° hydroxyl groups (3,3′). Thus, it would behave as a mono hydroxyl-free xanthophyll in the initial separation and would partition predominantly with the free xanthophylls. In support of this hypothesis, TLC separation of the pigments in the three fractions generally showed the neoxanthin band absent or very faint in the esterified xanthophyll fraction as compared to the free xanthophyll fraction. Free xanthophylls from green fruit skin had a spectral shape similar to the esterified xanthophyll fraction of yellow tissue (Fig. 3B), with a peak I at approximately the same absorption as the 457 nm baseline (data not shown).

Studies which utilized a mild saponification procedure that removed chlorophyll, but left the xanthophyll esters intact, confirmed the quantitative differences in esterified xanthophylls between the yellow and green tissue. The non-polar pigment from green fruit skin included a small amount of α-carotene (peak 1), a large peak of β-carotene (peak 2), a pigment tentatively identified as 5,6,5′,6′-diepoxy β-carotene (peak 3), and two minor peaks, designated A and B (Fig. 4). When yellow fruit skin was similarly analyzed the major pigments were A and B (Fig. 4B). Based on acetylation tests and partition behavior (Table 4) these

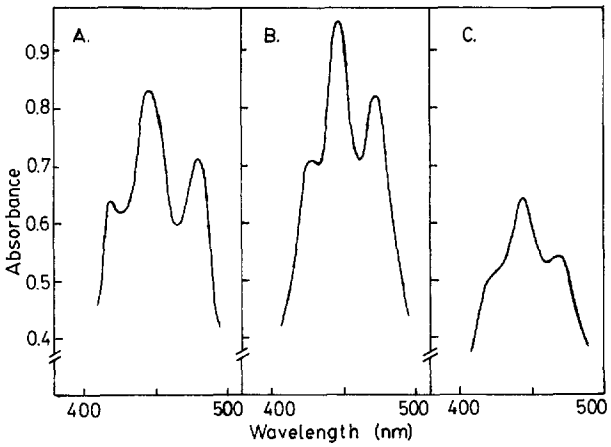

Fig. 3A–C. Absorption spectra of carotenoid fractions isolated from the skin of Early Prolific B^+B^+ *YY* fruit at 20 days after anthesis. **A** Free xanthophylls; **B** Esterified xanthophylls; **C** Carotenoids

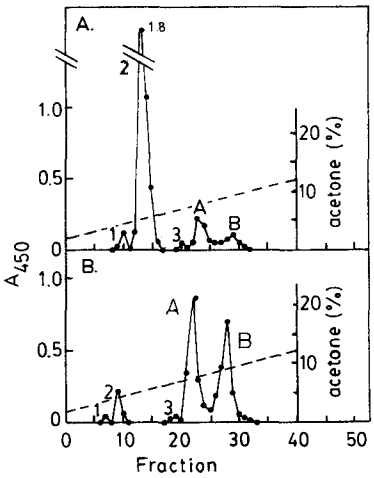

Fig. 4 A, B. Elution profiles from MgO : Hyflo Super-Cel columns of non-polar carotenoids isolated from the skin of FZ fruit harvested at 20 days after anthesis. Pigments were eluted with increasing acetone (2 to 12%) in petroleum ether. **A** FZ B^+B^+ *yy*; **B** FZ *BB yy*

Table 4. Partition coefficients and free OH-groups of standard carotenoids (from Purcell 1958) and peaks "A" and "B" of Fig. 4

Carotenoid	Free –OH groups	Partition coefficient, light petroleum – 95% methanol
β-Carotene	0	INF.[a]
Lycopene	0	INF.
Cryptoxanthin	1	4.46
OH-α-Carotene	1	2.26
Lutein	2	0.11
Violaxanthin	2	0.05
"A"	0	64.40
"B"	1	9.73

[a] INF = infinite

peaks were identified as esterified xanthophylls with no free OH- groups (peak A) and one free OH-group (peak B).

The different xanthophylls in each group did not completely separate on MgO: Hyflo Super-Cal columns (Fig. 4). However, in each peak (A and B), the first few fractions appeared to be violaxanthin esters, based on spectra and positive tests for two epoxides. The next fractions tested negatively for epoxides and apparently contained lutein, followed by the mono-epoxides, taraxanthin and neoxanthin, when present. There was an overlap in certain fractions giving intermediate spectra and epoxide test results but the order of elution coincides with the previously reported chromatographic behavior of xanthophylls on magnesia columns (Jungalwala and Cama 1962).

GC analysis of the fatty acids esterified to lutein isolated from yellow FZ *BB yy* skin at 30 days after anthesis showed lauric, myristic and palmitic acids in the ratio of 23 : 63 : 14 (Fig. 5). The identity of the fatty acids was confirmed by GC-MS. The number of different combinations of fatty acids esterified to xanthophylls resulted in a series of discrete bands for each xanthophyll on TLC.

Discussion

C. pepo skin chloroplasts resemble foliar chloroplasts with respect to pigment composition and relative proportions. This is in agreement with previous reports on green fruit of tomato (Rabinowitch et al. 1975), pepper (Davies et al. 1970) and orange (Eilati et al. 1975). *C. pepo* skin chromoplasts also contain foliar carotenoids but in different proportions, and with a high level of fatty acid-esterified xanthophylls. Similar results have been published for ripe yellow peppers (Davies et al. 1970) and ripe yellow apples (Knee 1972).

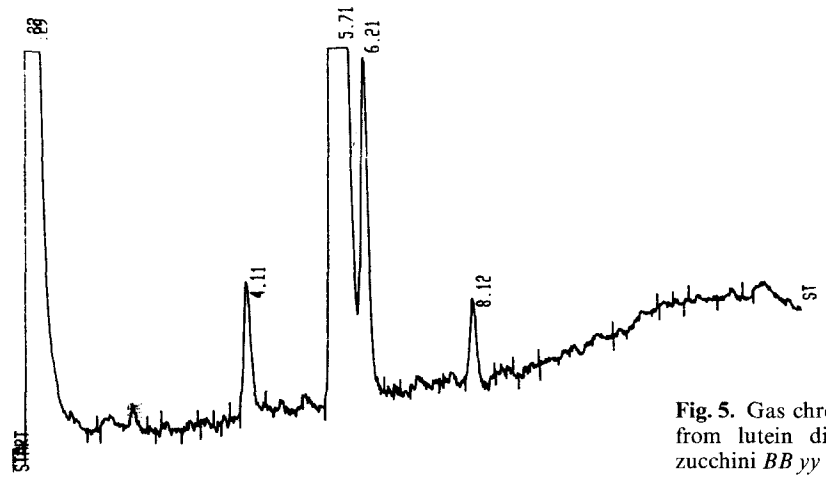

Fig. 5. Gas chromatograph profiles of fatty acid methyl esters from lutein diesters isolated from the skin of Fordhook zucchini *BB yy* fruit harvested at 30 days after anthesis

The results of this research indicate that *C. pepo* skin chromoplasts, regardless of their developmental and genetic origin, contain a very similar distribution of pigments. All the yellow chromoplast-filled tissues studied are characterized by a low carotene-to-xanthophyll ratio, and a high percentage of esterified xanthophylls.

FZ *BB yy,* at anthesis, is the only yellow tissue with a proportionally low level of esterification, perhaps indicating that it is in an intermediate stage of proplastid to chromoplast development. During the maturation of chromoplasts osmiophilic globules increase, and these apparently contain esterified xanthophylls (Eilati et al. 1977). Similarly, the increase in xanthophyll esterification in the green FZ B^+B^+ *yy* may be either an indication of chloroplast maturation, or chloroplast to chromoplast conversion due to senescence. At 60 days past anthesis chromoplasts are present in FZ B^+B^+ *yy* skin, particularly in the lower cell layers of the hypoderm is (Schaffer and Boyer 1983 b). Some of these plastid interconversions may have occurred by 20 days past anthesis, although they were not observed.

The low percentage of hydrocarbons in *C. pepo* chromoplasts apparently is characteristic of chromoplasts that contain primarily foliar carotenoids and that have originated from chloroplasts (Davies 1976; Eilati et al. 1975; Knee 1972). Hydrocarbons are located primarily in chloroplast thylakoid membranes (Douce and Joyard 1979), while xanthophylls are the main carotenoids of the plastid envelope (Douce and Joyard 1979; Fishwick and Wright 1980), and presumably in the plastoglobules. Thus, the decrease in hydrocarbons may be accounted for partially by the loss of thylakoid membranes, and preservation of the plastid envelope and plastoglobules, during chloroplast-to-chromoplast transformation.

The similarities between all *C. pepo* skin chromoplasts suggest that neither gene *B* nor gene *Y* affect carotenoid biosynthesis per se, but rather that these genes act at the level of plastid transformation. *B* has no effect on carotenoid pigments at maturity in EP, and previous studies have shown that in a number of other genotypes that normally are yellow due to *Y,* there is no effect of *B* on mature fruit skin color (Schaffer and Boyer 1984). Only in non-yellowing lines such as FZ does *B* manifest itself in fruit color at maturity. The decrease in total carotenoids in FZ *BB yy* as compared to FZ B^+B^+ *yy* also be a secondary effect of *B,* namely reduced fruit growth and development (Schaffer and Boyer 1984) rather than a direct consequence of *B* on plastid pigment biosynthesis. The similarity of patterns of pigment increase through development within each of the cvs. studied, regardless of *B,* also indicates that *B* does not act at the pigment biosynthesis level. Both genotypes of EP are characterized by linear increases, while both genotypes of FZ have lag periods between 20 and 30 days past anthesis.

In light of the results presented and the ultrastructural studies of Grilli (1965 a, b), Devide (1970), and Lijubesic (1970, 1972). *B* appears to act at the proplastid level to cause direct proplastid-to-chromoplast maturation. B^+B^+ allows for proplastid-to-chloroplast development, and *Y* later controls the time of chloroplast-to-chromoplast differentiation (Fig. 6).

Accordingly, EP B^+B^+ *YY* forms chloroplasts prior to anthesis, but these are transformed to chromoplasts due to *Y.* EP *BB YY* differs only by not first developing chloroplasts. However, EP *BB YY* chromoplast carotenoids are very similar to those of the B^+B^+ genotype. In addition, the total skin carotenoids of EP are similar, regardless of the allelic state at *B.* In EP, then, *B* affects the timing and origin of chromoplast; the final product, the chromoplast, is unaffected.

FZ B^+B^+ *yy* develops and maintains typical chloroplasts, as indicated by light microscope observations, carotenoid fraction profiles, and the level of xanthophyll esterification. FZ *BB yy* develops chromoplasts by anthesis, characterized by the absence of chlorophyll, and by the typical chromoplast carotenoid profile and esterification levels. Both genotypes have high carotenoid levels and *B* determines that the plastids of the FZ genotype will develop as chromoplasts.

Using the proposed terminology of Shifriss (1981) *Y* and *B* may be considered "timer" genes. However, they appear to be qualitatively different, one (*B*) working at the proplastid level and the other (*Y*) at the chloroplast level. A series of alleles which affect the timing of plastid conversion, (Schaffer 1982; Schaffer and Boyer 1984; Shifriss 1981), and a series of alleles of *B* which affect the amount of tissue precociously pigmented, (Shifriss 1981) have been identified.

From the results presented, however, it appears that chromoplasts derived from chloroplasts differ to some degree from chromoplasts derived from proplastids. Although EP B^+B^+ and *BB* chromoplast carotenoids were very similar they do differ at the three stages studied with respect to percent xanthophyll esterification. At 10 days past anthesis, EP B^+B^+ also had significantly more carotenoids than EP *BB* (Table 2), and this may be due to its chloroplast origin. In addition, EP *BB* and FZ *BB* chromoplasts, both derived directly from proplastids, have almost identical carotenoid fraction patterns. The susceptibility of non-green *C. pepo* fruits to regreening after maturity also points to qualitative difference between *BB* and B^+B^+ chromoplasts. Devide and Ljubesic (1971, 1974) have studied chromoplast to chloroplast reversions in *C. pepo* fruit, but have only de-

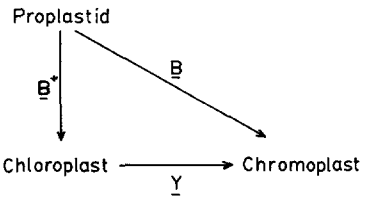

Fig. 6. Proposed effect of genes *B* and *Y* on plastid development in *Cucurbita pepo* L. fruit skin

scribed the phenomenon in tissue that had previously been green and not in the yellow portions of bicolored fruit. We also have found that certain B^+B^+ genotypes are susceptible to regreening, but do not regreen when carrying the *B* allele (Schaffer and Boyer 1983a). This implies that *B* not only directs proplastid to chromoplast development, but also that *B* derived chromoplasts are incapable of converting to chloroplasts. *B*, then, may alternatively be considered a chloroplast development inhibitor.

Shifriss (1981), observed that *B* increases total flesh carotenoids in *C. pepo,* in contrast to our results. This may be due to differential action of *B* in flesh and skin tissues. Grilli (1965a, b) observed that flesh and skin chromoplasts of *C. pepo* have different plastid origins. In the skin, *B* is responsible for chromoplast development at the expense of chloroplast development. Both plastids are highly pigmented, thus the effects on total carotenoid level are small. However, in the flesh, *B* increases chromoplast development at the expense of amyloplast development, thereby increasing carotenoid content. According to this hypothesis the starch content of *B* flesh would be expected to be less than in B^+B^+ flesh, although the influence on *B* on flesh pigmentation during development remains to be determined.

B appears to be a unique gene in terms of chloroplast development. Generally, nuclear genes that affect chloroplast development also significantly decrease carotenoid levels, and produce albino or light yellow phenotypes (Kirk and Tilney-Basset 1978; Robertson et al. 1978; von Wettstein 1961). In addition, most of these mutations also allow some chlorophyll to be synthesized. *B,* on the other hand, bypasses chloroplast development and produces what appear to be normal chromoplasts. Continued study of the *B* gene, particularly in terms of its action at the molecular level, should shed light on the complex and colorful subject of plastid development and genetics, in relation to fruit pigmentation of *C. pepo.*

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