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PIGMENT EVOLUTION IN *LYCOPERSICON ESCULENTUM* FRUITS DURING GROWTH AND RIPENING

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Abstract—During growth and subsequent maturation, the distribution and formation of pigments in the inner pulp and in the outer region of the pericarp of 'cherry' tomatoes is different. The foliar pigments (β-carotene, lutein, violaxanthin, auroxanthin, neoxanthin, chlorophylls *a* and *b*) accumulate in both parts of the growing fruit, then remain fairly constant, except for the chlorophylls and neoxanthin which decrease. Phytofluene and lycopene appear only at the end of ripening and almost exclusively in the external part of the fruit; lycophyll and lycoxanthin can be detected during the entire growth and maturation period. The results suggest that development involves three distinct steps and show that the growth period is separated from that of maturation by a lag phase, during which new regulatory phenomena occur and induce pigment transformations which lead to complete maturity.

INTRODUCTION

The study of carotenoid biosynthesis and control mechanisms in flowers [1,2] and fruits [3–7] is well documented. Although the ripening tomato fruit has often been the material of choice in these studies [8–17], no apparent effort has been made to separate the two physically distinct regions of the pericarp: the inner jelly-like portion containing the seeds and the outer portion of firm consistency. Several recent publications have dealt with pigment evolution and ultrastructure in ripening tomatoes [18–21], but again the pericarp was not separated.

In a previous study, we presented evidence of a morphological difference between the chromoplasts of the two regions of cherry tomato pericarp here called the inner "pulp" and outer "flesh". The present studies were undertaken to examine the possible differences in pigment composition and evolution in these two regions of the growing and ripening fruit. The results presented complement previous data on the relationship between pigment content and ultrastructure of plastids [22–23].

RESULTS

Pigment distribution between the "pulp" and "flesh" in normal and high temperature-ripened fruits

Based on the analyses of pigment content of leaves and of the two regions of the fruit (Table 1) we can distinguish three categories of pigments. (i) Exclusively foliar pigments: we have never detected flavoxanthin in tomato fruits at any stage of development. (ii) Common pigments:

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Table 1. Pigment composition of the inner "pulp" and outer "flesh" of "cherry"

‡	Phytofluene		Lycophyll and Lycoxanthin		Lycopene		β-Carotene		Lutein		Epoxylutein	
"Pulp"	*	†	*	†	*	†	*	†	*	†	*	†
6	0	0	4	0·3	0	0	78	5·5	70	4·9	0	0
8	0	0	4·5	0·2	0	0	67	3·5	132	7	0	0
10	0	0	2·5	0·2	0	0	91	6	126	8·3	0	0
12 or a	0	0	6	0·5	0	0	92	7·9	53	4·6	0	0
b	0	0	6·5	0·7	0	0	114	13·3	46	5·4	0	0
c	0	0	7·5	1·2	4	0·6	119	19	49	7·8	0	0
d	0	0	9·5	1·9	14	2·9	117	24	34	7	0	0
e	0	0	25	5·2	36	7·4	136	28	46	9·5	0	0
f	0	0	21	4·4	107	22·3	190	39·7	36	7·5	0	0
g	0	0	25	4·4	233	40·9	236	41·4	43	7·5	0	0
30°C§	0	0	0	0	0	0	140	48·3	107	36·9	13	4·5
"Flesh"												
6	0	0	3	0·2	0	0	67	3·6	84	4·5	0	0
8	0	0	6	0·4	0	0	100	7·6	130	10	0	0
10	0	0	5	0·3	0	0	100	6·7	125	8·4	0	0
12 or a	0	0	9	0·9	0	0	91	8·8	64	6·2	0	
b	0	0	8·5	1·1	0	0	84	10·8	78	10	0	0
c	14	2·3	13	2·2	32	5·4	123	20·6	64	10·7	0	0
d	74	9·4	21	2·7	244	31	164	20·8	47	6	0	0
e	129	10·7	21	1·6	820	64·5	187	14·7	32	2·5	0	0
f	131	8·4	21	1·3	1220	77·8	120	7·6	40	2·6	0	0
g	219	7·2	30	1	2530	82·9	209	6·8	33	1·1	0	0
30°C§	0	0	0	0	112	32	158	45·1	44	12·6	16	4·6
"Leaves"	0	0	0	0	0	0	760	5·3	705	4·9	0	0

For each fruit determination 40–50 fruits were harvested, mechanically separated into "pulp" and "flesh" and subjected to pigment analysis as described in methods. Analyses of fruits matured at 30° were performed when the fruits exhibited no further colour changes. Adult specimens were used for pigment analyses of leaves.

chlorophylls *a* and *b* were found in all tissues grown at normal temperature, even in fruits at the terminal stage of ripening, as were β-carotene, lutein and violaxanthin. Neoxanthin was found in leaves and in young fruits. (iii) Non-foliar pigments, identified in one or both regions of the fruit: phytofluene was found only in the "flesh", while lycopene was detected in both regions of the ripening fruit. Lycophyll and lycoxanthin, previously identified only occasionally [1,24], were detected in both regions of normally ripened fruits. Neither α-, ξ- or γ-carotene [25] nor δ-carotene [26], previously reported in some varieties of tomato were not detected. Although δ-carotene is considered the immediate precursor of β-carotene, its apparent absence does not necessarily imply another biosynthetic pathway for β-carotene, but perhaps an extremely small free pool of the precursor.

Excised fruits at the fully green stage and ripened at 30° exhibit particular pigment distribution at the fully mature stage. Lutein and β-carotene, and the violaxanthin + auroxanthin fraction were present at about the same levels as those in normally matured fruits. Lycophyll + lycoxanthin, and phytofluene were not detected, and chlorophyll is undetectable in the "flesh". Lycopene is present only in the "flesh" and in reduced quantities, consistent with previous reports of high temperature inhibition of lycopene synthesis [19].

Neoxanthin, not found in normal mature fruits, apparently persists and epoxylutein, a pigment not normally found in any tomato tissue we examined, is present in both regions of high temperature-ripened fruits. This suggests that at 30° the tissues exist in a more oxidized state [27] and these results are similar to those obtained with the avocado fruit [28].

Quantitative evolution of pigments

Table 1 allows the distinction of two groups of pigments according to their maximum accumulation in the fruit. Major pigments (>100 nmol/g

tomato fruits during growth and subsequent ripening, and of adult leaves

Violaxanthin		Violaxanthin and Auroxanthin		Flavoxanthin		Neoxanthin		Chlorophyll *a*		Chlorophyll *b*		Total pigments	
*	†	*	†	*	†	*	†	*	†	*	†	*	†
21	1·5			0	0	6	0·4	880	62·2	355	25·1	1414	99·9
53	2·8			0	0	16	0·8	1455	77·1	160	8·5	1887	99·9
		64	4·2	0	0	36	2·3	855	56·4	340	22·4	1514	99·8
		15	1·3	0	0	4	0·3	668	57·5	324	27·9	1162	100
		35	4·1	0	0	0	0	448	52·4	206	24·1	855	100
		31	5	0	0	0	0	291	46·7	122	19·6	623	99·9
		31	6·3	0	0	0	0	206	42·3	76	15·6	487	100
		45	9·3	0	0	0	0	144	29·7	53	10·9	485	100
		44	9·2	0	0	0	0	65	13·6	16	3·3	479	100
		33	5·8	0	0	0	0	traces	0	traces	0	570	100
		21	7·2	0	0	9	3·1	traces	0	traces	0	290	100
25	1·3			0	0	6	0·3	1455	77·4	239	12·7	1879	100
47	3·6			0	0	21	1·6	723	55·3	280	21·4	1307	99·9
		73	4·9	0	0	27	1·8	845	56·7	316	21·2	1491	100
		45	4·4	0	0	7	0·7	576	56	296	22·9	1028	99·9
		67	8·6	0	0	0	0	374	48·1	166	21·4	777	100
		48	8	0	0	0	0	215	36·1	87	14·6	596	99·9
		31	3·9	0	0	0	0	151	19·2	55	7·0	787	100
		16	1·2	0	0	0	0	49	3·8	18	1·4	1272	99·8
		31	2	0	0	0	0	3	0·2	1	0	1567	99·9
		29	0·9	0	0	0	0	traces	0	traces	0	3021	99·9
		15	4·3	0	0	5	1·4	0	0	0	0	350	100
90	0·6	0	0	30	0·2	44	0·8	11109	61·8	5587	26·8	19917	99·9

* nmol/g dry wt. † percentage of total pigment. ‡ Numbers are the dia (mm) of green growing fruits, the letters refer to stages of maturation: a: green fruit, 12 mm dia; b: yellow–green; c: yellow–pink; d: orange; e: pale red; f: red; g: deep red. § Fruits ripened at 30° were excised at the 12 mm dia stage.

dry wt) include chlorophylls *a* and *b* and lycopene, whose minima approach or are equal to zero, and β-carotene and lutein, which exhibit less profound fluctuations. Minor pigments (<100 nmol/g dry wt) include violaxanthin, auroxanthin, neoxanthin, lycophyll and lycoxanthin. Neoxanthin is the only minor pigment not detectable at certain stages of the life of the fruit. It is interesting to note that, with the exception of the non-foliar lycophyll + lycoxanthin fraction, the qualitative and quantitative pigment content of young green fruits closely resembles that of the leaf.

β-Carotene accumulates in both regions throughout the life of the fruit, its rate of accumulation being accelerated at the terminal stages of ripening. In both regions of the fruit lutein attains an early maximum and then declines to about half its initial level by the end of maturation. Phytofluene and lycopene both appear at the yellow pink stage (c) although the former is restricted to the "flesh". In this same region chlorophyll *a* decreases regularly during fruit evolution whereas a definite peak is noted in the "pulp". Chlorophyll *b* accumulation in the "flesh" reaches a peak toward the end of fruit growth while it apparently undergoes greater fluctuations in the "pulp".

The evolution of minor pigments is similar in both regions of the fruit. Pure violaxanthin is found only until the 8 mm dia stage and is thereafter found in the violaxanthin + auroxanthin fraction (see Experimental). Although their individual evolutions were not evaluated, the mixture evolves in the same manner in both regions of the fruit.

The fruit as a biological unit

The results presented in Table 1 are expressed on a dry wt basis in order to facilitate comparison with other published data. It was of interest to monitor the evolution of fr. and dry wt in the maturing fruits and the following results were obtained. In the outer "flesh" both fr. and dry wt evolve in parallel, the most active period of increase being between the yellow green (b) and

orange (d) stages. At the end of maturation the dry wt of the "flesh" is *ca* twice that at the onset of ripening, indicating that *de novo* syntheses occur or that material is translocated from other parts of the plant. In the inner "pulp", changes in dry wt are more important than those in fr. wt. These data on dry wt changes are in opposition to other published data [29] but this may be due to a species difference.

DISCUSSION

Our data on pigment content of the two regions of the pericarp during growth and maturation of cherry tomatoes [22] (Table 1) show that the "pulp" and "flesh" undergo different patterns of pigment biogenesis. This is consistent with the dimorphism of chromoplasts observed in these two zones [23]. We believe the analytical data of Table 1 are rendered more physiologically meaningful by extrapolation to the mean fruit as biological reference unit since changes in absolute pigment content could be masked by concomitant changes in dry wt. Thus, it is possible to separate the life of the fruit into three distinct phases namely, growth, transition and maturation.

In the young fruit the pigments are of the foliar type, as observed in other species [28–30], with minor differences. Fruits at this stage are slightly richer in hydroxylated alicyclic carotenoids (neoxanthin, violaxanthin, lutein) and already contain some lycophyll and lycoxanthin, exclusively non-foliar pigments. The growth of the young fruit (6 mm dia) to the fully green (stage a) is accompanied by a 10-fold enrichment in pigments in both regions of the pericarp. There are kinetic differences in chlorophyll accumulation which might be related to differences in photosynthetic capacity of these tissues, but in general, pigment distribution in the two zones during growth is similar.

The transition period, which we define as between the fully green and yellow pink stages (a–c), involves important pigment modifications in both regions of the fruit. It is during this period that the chlorophyll–carotenoid ratio declines from five to about one, accompanied by a disorganization of the granal system [23] as previously observed [31–33].

The transition period in the tomato fruit is comparable to leaf senescence [5] where the disappearance of chlorophyll is accompanied by a lowered carotenoid content compared to green leaves. The photodestruction of chlorophyll would be favored by a lowered carotenoid production [15], especially xanthophylls as recently found [34], and is associated with a morphological disorganization of the chloroplasts. The relation between the pigment composition (chlorophyll–carotenoid ratio, membranal association between chlorophylls and β-carotene) [35–37] and chloroplast structure [38] (photosynthetic membrane integrity) is probably one of the most affected factors, during the transition period.

During transition, total pigment per fruit declines in both regions, largely as a result of the disappearance of chlorophylls *a* and *b*, as observed in other systems [39]. During this period there is only *a* slight accumulation of β-carotene, suggesting that if phytol groups resulting from chlorophyll degradation are eventually used for carotenoid biosynthesis [12], there would exist during transition a substantial free pool of phytol awaiting subsequent re-utilization.

During the maturation phase of the fruit the total pigment content does not appreciably change in the "pulp", consistent with the possible re-utilization of phytol [12] already discussed and relatively little new material accumulates. In the "flesh", however, the *ca* 4-fold increase in carotenoid content, represented largely by lycopene in typical tomato chromoplasts [18–21,23,31–33] implies a significant formation of new material. During maturation β-carotene and lycopene accumulate in the fruit, consistent with the latter being the precursor of the former [8,9,40,41]. On the other hand, we find no lycopene in fruits ripened at 30° or in leaves whereas significant β-carotene is present in these tissues. This suggests another route for β-carotene synthesis [11,42,43] as does the different ratio of lycopene/β-carotene in the two regions of the fruit. Small pools of lycopene undergoing rapid turnover cannot be excluded.

In the apparent absence of chlorophyll (and presumably phytol) synthesis during maturation it is conceivable that geranyl-geranyl-PP could be diverted towards carotenoid biosynthesis [1,44]. In this context it is interesting to note that in

certain systems the inhibition of chlorophyll accumulation is associated with an accumulation of lycopene in typical chromoplast structures, even in tissues not normally containing this carotenoid [45]. In our material we cannot eliminate, however, the possibility that phytol is still produced during maturation and is transformed into carotenoids, as demonstrated in other systems [12].

We have demonstrated that pigment metabolism during ripening of cherry tomato fruits is qualitatively and quantitatively different in the two regions of the pericarp. The inner "pulp" is relatively inactive and whether this difference is caused by genetic or environmental factors, or both, we conclude that a definite compartmentalization exists. These metabolic differences are deranged in fruits excised at the fully green stage and ripened at 30°, the pigment content of the two regions generally resembling either young fruits or mature "pulp". Thus, pigment evolution characteristic of the "flesh", especially lycopene formation, is highly temperature sensitive. Clearly pigment formation in this material is under genetic control, as demonstrated in other systems [46–48] and the consequent enzymatic steps [17,41,49] are expected to be temperature sensitive [19,27,50]. We cannot say if alterations in pigment metabolism at 30° are due to changes at the transcriptional or translational level, or at the level of individual enzyme activity. Changes in the permeability of the plastid envelope [40–42], especially toward mevalonate cannot be excluded.

EXPERIMENTAL

Lycopersicum esculentum var. *cerasiforme* (DUN) A. Gray were grown under natural conditions. Fruits were classified by size during growth (6–12 mm dia) and by color during maturation, as previously described [21]. For each determination 40–50 fruits were harvested and divided into the external region of the pericarp (called the "flesh" and having a rather firm consistency) and the fluidic internal portion (called the "pulp" and including the seeds). The separated materials were immediately frozen in liquid N_2, lyophilized and stored *in vacuo* in darkness before analysis.

Pigment extraction and separation. Lyophilized material was powdered in a ball grinder in the presence of $CaCO_3$ and liquid N_2. All subsequent operations were performed at $\leqslant 4°$. Pigments were extracted with Me_2CO–H_2O (9:1) and an aliquot of this extract, corresponding to 250 mg of dried tissue, was saponified, washed, evaporated and finally dissolved in petrol (40–60°) containing 2% Me_2CO. In the case of dark red fruits the "flesh" still contained pigments after the first extraction. The product of a second extraction with hexane yielded only one chromatographic fraction (see below) with an absorption spectrum of lycopene.

A preliminary separation of carotenoids was obtained by ascending chromatography on a cellulose powder column [51]. Pigments were eluted with a gradient of Me_2CO (2–10%) in petrol. The first fraction eluted contained a mixture of carotenoids which was further fractionated with the same solvent system by descending chromatography on $Ca(OH)_2$ in a stainless steel column operating under a N_2 pres. of 3 kg/cm^2.

Identification and estimation of pigments. Chlorophyll *a* and *b* content in the original Me_2CO–H_2O extract was determined by controlled conversion to pheophytin products [52,53]. Carotenoids and their derivatives were identified and estimated by conventional methods [54,55]. The different fractions obtained by the above methods were estimated by reference to the main component. When auroxanthin, which elutes with violaxanthin, was present, the two carotenoids were estimated together as violaxanthin. Since lycoxanthin was always present in the lycophyll fraction, these two carotenoids were also estimated together as lycophyll. Chlorophyll and carotenoid determinations were reproducible to within ±3% [56]. Pigments extracted from lots of five tomatoes showed a variability of ±2·5% for green fruits and ±24% for mature specimens [57]. We minimized this apparent heterogeneity by sampling 40–50 fruits, corresponding to 10 g dry wt, for each determination which only required 250 mg of material.

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