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Carotenoid Biosynthesis during Tomato Fruit Development[1]

Evidence for Tissue-Specific Gene Expression

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Tomato (*Lycopersicon esculentum* Mill. cv Ailsa Craig) fruit, at five stages of development, have been analyzed for their carotenoid and chlorophyll (Chl) contents, in vitro activities of phytoene synthase, phytoene desaturase, and lycopene cyclase, as well as expression of the phytoene synthase (*Psy*) and phytoene desaturase (*Pds*) genes. During ripening, the total carotenoids increased with a concomitant decrease in Chl. Although the highest carotenoid content (consisting mainly of lycopene and β-carotene) was found in ripe fruit, the greatest carotenogenic enzymic activities were found in green fruit. Phytoene synthase was located in the plastid stroma, whereas the metabolism of phytoene was associated with plastid membranes during all stages of fruit development. The in vitro products of phytoene desaturation altered from being predominantly phytofluene and ζ-carotene in chloroplasts to becoming mainly lycopene in chromoplasts. The expression of *Psy* was detected in breaker and ripe fruit, as well as flowers, but was not detectable by northern blot analysis in leaves or green fruits. The *Pds* gene transcript was barely detectable in green fruit and leaves but was expressed in flowers and breaker fruit. These results suggest that transcription of *Psy* and *Pds* is regulated developmentally, with expression being considerably elevated in chromoplast-containing tissues. Antiserum to the *Synechococcus* phytoene synthase cross-reacted with phytoene synthase of green fruit only on western blots and not with the enzyme from ripe fruit. In contrast, a monoclonal antibody to the *Psy* gene product only cross-reacted with phytoene synthase from ripe fruit. The enzymes from green and ripe fruit had different molecular masses of 42 and 38 kD, respectively. The absence of detectable *Psy* and *Pds* mRNA in green tissues using northern blot analyses, despite high levels of phytoene synthase and desaturase activity, lends support to the hypothesis of divergent genes encoding these enzymes.

The ripening of tomato (*Lycopersicon esculentum*) fruit is a regulated process during which the color, flavor, aroma, and texture change in a coordinated manner. One of the most noticeable characteristics of ripening is the dramatic increase in the carotenoid content of the fruit (Laval-Martin et al., 1975). The change in pigmentation is caused by a massive accumulation of lycopene within the plastids and the disappearance of Chl. The chloroplasts of the mature green fruit change into chromoplasts, which accumulate lycopene in membrane-bound crystals (Harris and Spurr, 1969). Early biochemical studies of tomato mutant varieties were the basis on which the desaturase pathway from phytoene to lycopene was established (Porter and Lincoln, 1950), and Porter and co-workers used cell extracts from tomato fruit to study the properties of the enzymes (Porter, 1969; Porter and Spurgeon, 1979).

Carotenogenic enzymes of higher plants are located within the plastid (Kreuz et al., 1982; Lütke-Brinkhaus et al., 1982; Linden et al., 1993a), but their encoding genes are nuclear (Kirk and Tilney-Bassett, 1967). cDNA clones of two carotenoid genes from tomato have been characterized recently. A ripening-enhanced mRNA, represented by the cDNA pTOM5 (Maunders et al., 1987; Ray et al., 1992), has been shown to encode phytoene synthase (Bird et al., 1991; Bramley et al., 1992). This has been confirmed independently by Bartley and co-workers (1992), who have named the gene *Psy1*. The cDNA of the phytoene desaturase gene (*Pds*) has also been cloned and sequenced from a library prepared from ripening fruit (Pecker et al., 1992a).

The regulatory mechanisms that control carotenoid biosynthesis in plants are poorly understood, and the activities of carotenogenic enzymes in the plastids during fruit ripening have neither been documented nor correlated with the amounts and types of carotenoids in the fruit. Since the accumulation of lycopene in plants is unusual and does not occur in green tissue, it is possible that the regulation of carotenogenesis in green, chloroplastidic tissues is different from that in chromoplast-containing fruit. This makes the tomato fruit an ideal tissue in which to investigate carotenoid biosynthesis in relation to plastid differentiation.

To understand the changes that take place in the carotenoid content of tomato during fruit development and ripening, it is necessary to be able to estimate the activities of the enzymes catalyzing the formation of carotenoids and associated isoprenoids as well as the level of expression of the appropriate genes. In this study fruit at five stages of development have

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Abbreviations: d.p.b., days postbreaker; FDP, farnesyl diphosphate; GGDP, geranylgeranyl diphosphate; TBST, Tris-buffered saline/Tween.

been used to estimate the activities of carotenogenic enzymes, the presence of phytoene synthase in stromal fractions by western blots, as well as the detailed pigment content of the fruits, flowers, leaves, and roots, and the transcription of the phytoene synthase and phytoene desaturase genes.

MATERIALS AND METHODS

Radiochemicals

3*R*-[2-^{14}C]Mevalonolactone (53 mCi/mmol) and [4-^{14}C]IDP (56 mCi/mmol) were purchased from Amersham International. The former was converted to the sodium salt prior to use (Aung Than et al., 1972). [^{3}H]GGDP (15 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Gene Probes

The 1.64-kb pTOM5 cDNA was the same as that used in a previous study (Maunders et al., 1987). A 1.3-kb *Eco*RI fragment of the *Pds* cDNA from ripening tomato fruit (Pecker et al., 1992a) was kindly provided by Dr. J. Hirschberg (University of Jerusalem).

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill. cv Ailsa Craig) were grown in a greenhouse with supplementary lighting. Fruit were harvested at five stages of development: immature green (approximately 2 weeks after fertilization), mature green (approximately 7 weeks after fertilization), breaker, firm red (approximately 7 d.p.b.), and overripe (14 d.p.b.). Leaves were taken at expanding and fully expanded stages. Flowers were picked when fully open but before pollination, whereas roots were obtained from plants grown to approximately 1 foot in height. Pigment analyses were carried out immediately, whereas tissue for RNA extraction was frozen in liquid nitrogen. Fruit and leaves were left overnight in the dark and at 4°C in moist bags, prior to enzyme assays to reduce the starch content of the cells.

Fungi

The C5*carB10*(−) (phytoene-accumulating) and C9*carR21*(−) (lycopene-accumulating) strains of *Phycomyces blakesleeanus* were kindly provided by Professor E. Cerdá-Olmedo (University of Seville, Spain). The strains were cultured as described previously (Aung Than et al., 1972).

Extraction and Determination of Total Chl's and Carotenoids

Fresh tissue (about 0.25 g) was cut into small pieces and homogenized in methanol (3 mL) using an Ultra-Turrax blender (2 × 30 s). Pigments were extracted by partitioning (× 3) against 10% diethyl ether in light petroleum (boiling point 40–60°C). The dried lipid extract was redissolved in diethyl ether (1 mL), and the total carotenoids and Chl's were estimated in accordance with the method of Lichtenthaler and Wellburn (1983). Three separate determinations were made in each case, and values are reported as the means of the three determinations.

Separation and Determination of Carotenoids

Where necessary the Chl's were removed by saponification of total lipid extracts with 6% (w/v) KOH (Britton, 1991). Individual carotenoids were separated by HPLC on a reversed-phase C_{18} column (Spherisorb ODS1, 3 μm) using an eluting solvent of acetonitrile:methanol:isopropanol (85:10:5, v/v/v) at a flow rate of 1 mL/min. Carotenoids were monitored from their spectra with an on-line diode array detector (Bramley, 1992). The amount of each carotenoid was determined by integration of the peak area.

Isolation of Chloroplasts and Subchloroplast Fractions from Leaves

All procedures were carried out at 4°C. Leaf tissue was added to isolation buffer (50 mM Tricine [pH 7.9], 330 mM sorbitol, 2 mM EDTA, 0.1% BSA) in a ratio of 1:4 (w/v) and homogenized for 2 × 5 s in a Waring blender. The homogenate was filtered through two layers of muslin and one of cotton wool. The filtrate (30 mL) was layered onto a medium (14 mL) containing 40% (v/v) Percoll in isolation buffer. After centrifugation at 4000*g* for 5 min the intact chloroplasts were pelleted. The chloroplasts were broken in hypotonic lysis buffer (10 mM Hepes [pH 8.0] containing 5 mM DTT, 1 mM EDTA, and 0.5% Tween 60) containing a mixture of protease inhibitors (PMSF, 1 mM; leupeptin, 1 μg/mL; and pepstatin, 1 μg/mL). After 15 min the suspension was centrifuged at 105,000*g* for 1 h, and the resultant supernatant was used as the stromal fraction.

Isolation of Chloroplastidic Fractions from Green Tomatoes

Fruits were deseeded, cut into small pieces, and immersed in ice-cold 50 mM Tris-HCl buffer (pH 8.0), containing 0.4 M Suc and 1 mM EDTA (ratio 1:2, buffer:fruit). The mixture was homogenized for 2 × 3 s in a Waring blender and then filtered through two layers of muslin and one of Blutex (50 μm). The filtrate was centrifuged at 5000*g* for 10 min at 4°C. The loose pellet was resuspended in the above buffer (5 mL) and recentrifuged at 9000*g* for 10 min to yield the chloroplast preparation.

Stroma was obtained by homogenization of the resuspended chloroplasts with a hand-held Teflon homogenizer, followed by centrifugation at 105,000*g* for 1 h. The resultant supernatant was removed and either used immediately or frozen in liquid nitrogen. The pellet was resuspended in 0.4 M Tris-HCl buffer (pH 8.0), containing 5 mM DTT (one-third of the original volume), and used as the chloroplast membrane fraction.

Isolation of Chromoplast Fractions

Fruit were prepared and homogenized as described in the previous section. After the sample was filtered through Blutex, the extract was centrifuged at 2000*g* for 5 min. The supernatant was then centrifuged at 5000*g* for 10 min, and

the pellet was washed twice with extraction buffer. The washed chromoplast suspension (2 mL) was layered onto a stepwise Suc gradient (9 mL) containing 0.45, 0.84, and 1.45 M Suc in 50 mM Tris-HCl buffer (pH 7.6), containing 1 mM DTT. Following centrifugation at 62,000*g* for 1 h, intact chromoplasts were obtained at the interface between 0.84 and 1.0 M Suc.

Enzyme Assays

Phytoene synthase was assayed by measuring the incorporation of [1-^{14}C]IDP into phytoene. Incubations (500 μL) contained 0.5 μCi of [1-^{14}C]IDP, 350 μL of stroma, 4 μmol of $MgCl_2 \cdot 6H_2O$, 3 μmol of ATP, 6 μmol of $MnCl_2 \cdot 4H_2O$, 0.1% (v/v) Tween 60, 0.5 μmol of potassium fluoride in 0.4 M Tris-HCl buffer (pH 8.0) containing 5 mM DTT. Each incubation was at 25°C for 6 h. Phytoene was extracted from the incubations by partition with hexane and separated as described previously (Fraser et al., 1991).

Phytoene desaturase was assayed by measuring the incorporation of [^{14}C]phytoene into unsaturated carotenes using the coupled enzyme assay described in detail by Fraser and co-workers (1991). Incubations contained 200 μL of plastid preparations. Carotenes were extracted from the incubations by partition with hexane and then separated by HPLC using Spherisorb ODS1 (3 μm) with ethyl acetate:acetonitrile:water (35:60:5, v/v/v) as the eluting solvent at 1.0 mL/min.

Lycopene cyclase was assayed in plastid preparations by the incorporation of [^{14}C]lycopene into β-carotene (Bramley and Sandmann, 1985; Fraser, 1992). Carotenoids from the incubations were separated by HPLC on Spherisorb ODS1 (3 μm) using ethyl acetate:acetonitrile:water (35:60:5, v/v/v) at 1.0 mL/min.

Radioassay

Radioactive products on thin layers and HPLC were identified by co-chromatography with authentic standards. Radiolabeled compounds on thin layers were quantified by liquid scintillation counting (Bramley et al., 1974). Eluates off HPLC were quantified by an on-line radioactivity detector (Ramona; Lablogic Instruments, Sheffield, UK).

Immunological Techniques

Polyclonal antiserum against the purified phytoene synthase of *Synechococcus* PCC 7942 (Chamovitz et al., 1992) was raised in rabbits using standard protocols. Monoclonal antibodies were raised against a phytoene synthase/β-galactosidase fusion protein using a gene fusion with pTOM5 cDNA (P.D. Fraser, N. Misawa, G. Sandmann, and P.M. Bramley, unpublished data). Stromal proteins from fruit were subjected to SDS-PAGE (Laemmli, 1970), and the proteins were transferred by wet blotting onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Harrow, Middlesex, UK) for 1 h at 100 V in 25 mM Tris-HCl (pH 8.3) containing 192 mM Gly, 20% (v/v) methanol, and 0.01% (w/v) SDS. The blots were treated for 60 min with TBST, comprising 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% (v/v) Tween 20, containing 1% (w/v) BSA. The blocking solution was removed and the blot was incubated for 60 min with polyclonal or monoclonal antiserum at a dilution of 1:1000 with TBST. Unbound antiserum was removed by washing in TBST (3 × 5 min). The blots were then incubated for 1 h with anti-rabbit IgG-alkaline phosphatase (Promega), previously diluted 1:5000 in TBST. Following further washing in TBST (3 × 5 min) and TBS (2 × 1 min), visualization of the immunoreaction was performed by adding western blue-stabilized substrate for alkaline phosphatase until the desired intensity was obtained. The reaction was stopped by washing in deionized water (2 min). Mol wt values were obtained from markers (Rainbow markers, Amersham International). Control blots contained preimmune serum, diluted 1:5000 in TBST.

Immunoinhibition assays were performed on stromal fractions, preincubated with the polyclonal antiserum (5–20 μL) for 20 min at room temperature, followed by the addition of the standard assay mixture for phytoene synthase as described above. Immunoprecipitation of enzyme activity involved the addition of antiserum (0–40 μL) to the stromal fractions for 20 min at room temperature, followed by incubation overnight at 4°C. Protein A (5 mg), previously equilibrated in 0.75 M NaCl, 0.1% Tween 60, 0.5% (w/v) BSA, buffered with 20 mM Tris-HCl (pH 7.8), was then added to the mixture and incubated for 1 h at room temperature. The suspension was centrifuged for 5 min at 12,000*g*, and the supernatant was assayed for phytoene synthase activity. Control reactions were performed under the same conditions with preimmune serum.

Extraction and Analysis of RNA

RNA was extracted from tomato pericarp and leaves as described previously (Smith et al., 1990). Flower RNA was obtained using the protocol of Jepson and co-workers (1991). In all cases RNA was fractionated on 1.5% agarose gels containing formaldehyde (7%) and blotted onto nylon membranes (Hybond N, Amersham International). The membranes were hybridized with randomly primed ^{32}P-labeled cDNAs as described previously (Smith et al., 1990).

Other Determinations

Protein contents of cell extracts were determined in triplicate by the method of Lowry and co-workers (1951).

RESULTS

Carotenoid Content of Fruits, Leaves, and Flowers

During the development of the tomato fruit from immature green to fully ripe (14 d.p.b.), major changes in pigmentation were observed (Fig. 1A). The Chl content was maximal in immature fruit and decreased dramatically as fruit developed and ripened. The ripening process was accompanied by a rapid increase in the carotene content of the fruit (53-fold), with an approximate 10-fold decrease in the xanthophyll level as a proportion of total carotenoids. The amounts of individual carotenoids at three stages of fruit development and in flowers were determined by HPLC analysis as illustrated in Figure 2, where the characteristic pattern of chloroplastidic and chromoplastidic carotenoids are shown.

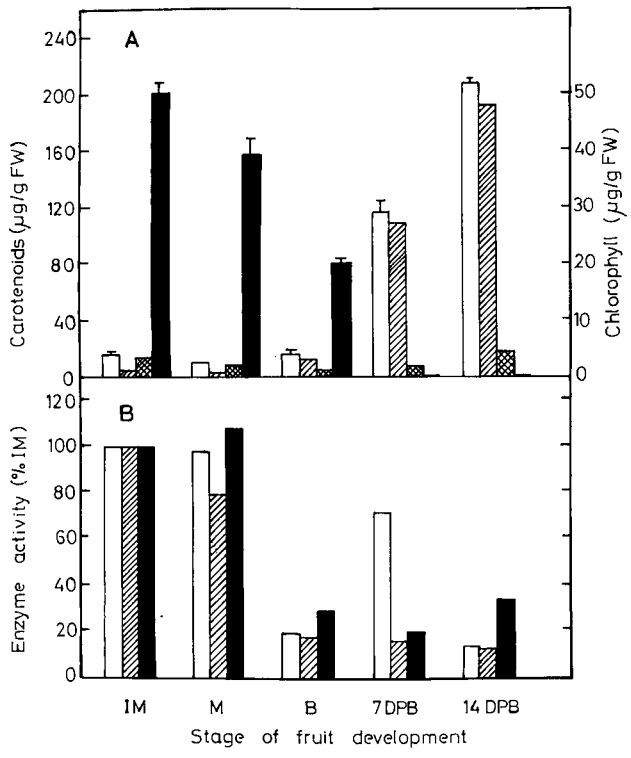

Figure 1. Alterations in pigment content and enzyme activities during tomato fruit development. Fruit at five stages of development (IM, immature green; M, mature green; B, breaker; 7 and 14 d.p.b.) were analyzed for pigments (A) as total carotenoids (□), Chl (■), carotenes (▨), and xanthophylls (cross-hatched columns). Carotenogenic enzyme activities (B) were estimated in plastids at each stage of fruit development: phytoene synthase (□), phytoene desaturase (▨), and lycopene cyclase (■). Values in B are expressed as the percentage of activities in immature fruit. Values for immature fruit were 880,000, 92,000, and 43,637 dpm/mg protein for phytoene synthase, phytoene desaturase, and lycopene cyclase, respectively. FW, Fresh weight.

The characteristic foliar carotenoids found in green fruit, i.e. lutein, neoxanthin, and violaxanthin, decreased as the ripening process progressed, whereas the lycopene and β-carotene contents were increased by 282- and 11-fold, respectively, at 14 d.p.b. (Table I). In addition, ripe fruit accumulated saturated carotenes such as two isomers of ζ-carotene, 15-*cis*-phytofluene, and especially 15-*cis*-phytoene and its epoxide. The carotene:xanthophyll ratio changed from 0.26 in immature fruit to 14.4 at 7 d.p.b., reflecting the abundance of carotenes in ripe fruit. Leaf pigments did not change in the same fashion during the change from expanding to expanded leaf, with the main change being an increase in β-carotene and neoxanthin levels. Flowers showed a more complex pattern of pigments (Fig. 2D). The roots contained only traces of foliar carotenoids.

Carotenogenic Enzyme Activities

The rapid and large accumulation of carotenes, particularly lycopene and β-carotene, from breaker stage onward was not reflected in an increase in the phytoene desaturase and lycopene cyclase activities in the same fruit. Both remained relatively constant throughout ripening (Fig. 1B). All of the carotenogenic enzymes assayed showed the greatest activities in green fruit, with significantly lower activities in breaker and postbreaker fruit. Only phytoene synthase activity increased (approximately 4-fold) during fruit ripening (7 d.p.b.) compared to the level in breaker fruit.

The product of the phytoene synthase reaction was 15-*cis*-phytoene in all cases with only negligible amounts (<1%) of the all-*trans*-isomer being found in vitro or in vivo. If mevalonic acid, FDP, or GGDP were used as substrates for phytoene synthesis, incorporations were only approximately 2% of those obtained with IDP (data not shown). A detailed analysis of the product profile of phytoene desaturation is shown in Table II. The patterns of conversion of phytoene into unsaturated carotenes differed with extracts from chromoplastidic tissue from those produced from chloroplasts; the former produced predominantly all-*trans*-lycopene, whereas the enzyme preparations from the latter converted phytoene into predominantly 15-*cis*-phytofluene and ζ-carotene.

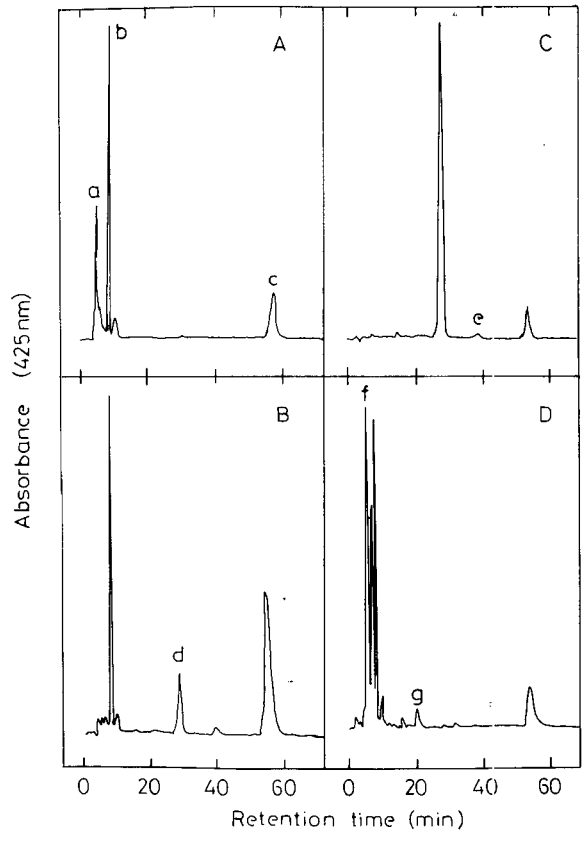

Figure 2. Separation by HPLC of major carotenoids from immature (A), breaker (B), 7-d.p.b. (C) fruit, and flowers (D). Pigments were extracted from fruits and flowers as described in "Materials and Methods" and separated by HPLC. Lipid extracts were saponified prior to chromatography. Pigments were identified by their retention times and absorption spectra and quantified by integration of peak areas. a, Neoxanthin; b, lutein; c, β-carotene; d, lycopene; e, γ-carotene; f, violaxanthin; g, β-carotene 5,6-epoxide.

Table I. *Carotenoid content of tomato tissues*

Fruit at five stages of development, leaf, flower, and root were analyzed for their carotenoid contents (see "Materials and Methods"). Abbreviations: P, 15-*cis*-phytoene; PF, phytofluene; ζ-Car I/II, ζ-carotene isomers; N, neurosporene; L, lycopene; γ-Car, γ-carotene; β-Car, β-carotene; Lut, lutein; Viola, violaxanthin; Neo, neoxanthin; P-X, phytoene epoxide; Lycop, lycophyll; Car, carotenes; Xan, xanthophylls. All values are μg/g fresh weight. tr = <0.1 μg/g fresh weight.

Tissue	Carotenes								Xanthophylls						Car: Xan Ratio	Total Carotenoid
	P	PF	ζ-Car I/II	N	L	γ-Car	β-Car	Total	Lut	Viola	Neo	P-X	Lycop	Total		
Fruit																
Immature green	0	0	0	0	0.25	0	3.3	3.55	9.2	0.9	3.4	0	0	13.5	0.26	17.1
Mature green	0	0	0	0	0.1	0	2.1	2.2	4.7	1.7	1.5	0	0	7.9	0.28	10.1
Breaker	1.9	0	0	0	3.7	0.4	5.6	11.6	1.5	0.4	1.1	0	1.3	4.3	2.70	15.9
4 d.p.b.	9.5	1.9	22.0	0	46.0	3.5	25.3	108.2	3.8	0.9	0.9	0.6	1.3	7.6	14.4	115
24 d.p.b.	40.6	22.2	7.5	0	70.5	11.3	36.8	189	6.4	2.3	2.3	6.4	0.4	17.8	10.6	207
Leaf																
Expanding	0	0	0	0	4.7	0	96.3	101	311.8	6.0	21.5	0	0	339.3	0.30	440.3
Expanded	0	0	0	0	0	0	242.4	242.4	305.0	9.3	111.4	0	0	425.7	0.57	668.1
Flower	0	0	0	0	0	0	48.0	52.5[a]	135	263	0	0	0	409[b]	0.13	461.5
Root	0	0	0	0	0	0	tr	tr	0.27	tr	tr	0	0	0.27		0.3

[a] Includes 4.5 μg/g fresh weight α-carotene. [b] Includes 11.0 μg/g fresh weight hydroxy-β-carotene.

Intraplastidic Location of Phytoene Synthase

Phytoene synthase was located predominantly (>95%) in the plastid stroma in all fruit when the stroma was isolated from plastids in a high salt buffer (Table III).

Psy and *Pds* mRNA Levels

Quantification of the *Psy* transcript by northern blot analysis during fruit development revealed a different pattern from the in vitro enzymic activities of phytoene synthase. A 2.0-kb transcript was detected in breaker and postbreaker fruit but not in leaf, green fruit, or root. The highest levels of *Psy* steady-state mRNA were found in flowers. A 3-kb transcript from *Pds* gene was found in breaker and postbreaker fruit but not in leaf or green fruit. The highest level of *Pds* mRNA was in the breaker fruit (data not shown).

Table II. *Product profile of phytoene desaturation in tomato tissues*

Extracts were incubated with [^{14}C]phytoene (200,000 dpm) and the products analyzed by HPLC (see "Materials and Methods"). No activity was detected in root cell extracts. Abbreviations as in Table I.

Tissue	Total Activity	Distribution of Radioactivity in Phytoene Metabolites		
		PF	ζ-Car	L
	dpm/mg protein		%	
Fruit				
Immature green	86,857	33	44	23
Mature green	73,712	21	52	11
Breaker	15,691	15	0	85
4 d.p.b.	14,245	9	12.4	71
14 d.p.b.	9,417	12.5	7.1	80
Flower	87,306	4.0	9.9	86

Immunological Detection and Immunoinhibition of Phytoene Synthase Activity

To establish the presence and immunological properties of phytoene synthase in green and red tissue, two antibodies were used: a polyclonal antiserum raised against phytoene synthase of the photosynthetic cyanobacterium *Synechococcus* and a monoclonal antibody raised against phytoene synthase encoded by *Psy* of tomato fruit. When stromal fractions of leaf, mature green, and 4-d.p.b. fruit were western blotted with polyclonal antiserum prepared against phytoene synthase of *Synechococcus*, an immunoreactive band, M_r 42,000, was detected in leaf and green fruit but was absent from ripening fruit. A second band of M_r 40,000 was also present in the stroma from green fruit (Fig. 3). In contrast, western blots of stromal fractions with the monoclonal antibody raised against the Psy protein of tomato only reacted with a single protein (M_r 38,000) in ripening fruit but not with stroma from leaf or green fruit (Fig. 4). Phytoene synthase activity was immunoprecipitated from stroma of green but not 4-d.p.b. fruit by the *Synechococcus* anti-phytoene synthase serum (Fig. 5). This antiserum also inhibited phytoene

Table III. *Intracellular location of phytoene synthase in tomato fruit*

Stroma and plastid membranes from mature green, breaker, and 4- and 14-d.p.b. fruit were assayed for phytoene formation by incubation with [1-^{14}C]IDP. Values are expressed as dpm/mg protein; those in parentheses are percentages of total enzyme activity.

Tissue	Plastid Fraction	
	Stroma	Pellet
Mature green	103,293 (99)	1,168 (1)
Breaker	65,868 (96)	2,599 (4)
4 d.p.b.	3,628 (97)	105 (3)
14 d.p.b.	2,206 (96)	97 (4)

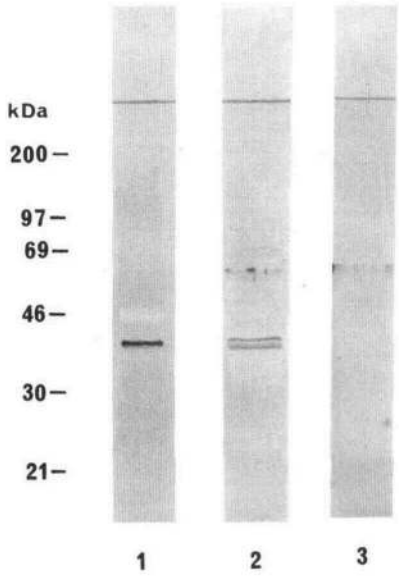

Figure 3. Western blot of stromal fractions of leaf (lane 1), green fruit (lane 2), and 4-d.p.b. fruit (lane 3), with antiserum raised against phytoene synthase from *Synechococcus*. Lanes contained 30 μg of protein. Molecular mass markers are shown on the left.

synthase activity in green but not ripe fruit stroma (data not shown).

DISCUSSION

The quantitative and qualitative changes in pigment content that were found during tomato fruit development (Fig. 1A; Table I) are indicative of a complex series of regulatory and metabolic events that occur during chloroplast development and the transition of mature chloroplasts into chromoplasts during fruit ripening. Four lines of experimental evidence indicate that the control of carotenoid biosynthesis is different in chloroplast and chromoplast-containing tissue: (a) different carotenoids accumulate in the two types of tissue, (b) the in vitro products of phytoene metabolism change after the transition of chloroplasts into chromoplasts, (c) the very low extent of expression of the *Psy* and *Pds* genes (cloned

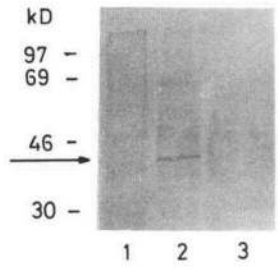

Figure 4. Western blot of stromal fractions of green fruit (lane 1), 7-d.p.b. fruit (lane 2), and leaf (lane 3) with monoclonal anti-phytoene synthase raised against an overexpressed pTOM5/β-galactosidase fusion protein. Each lane contained 25 μg of protein. Molecular mass markers are shown on the left.

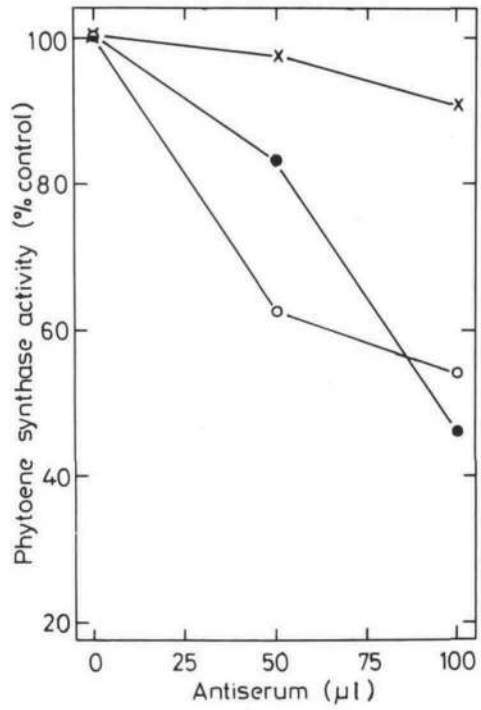

Figure 5. Immunoprecipitation of phytoene synthase activity from leaf (●), mature green fruit (○), and 7-d.p.b. fruit (×) stroma using antiserum to the *Synechococcus* phytoene synthase. Stromal fractions (25 μg of protein) were treated with increasing volumes of antiserum and diluted 1:1000, and the enzyme activity of the supernatant was assayed.

from ripening fruit) in green fruit is unlikely to support the high levels of carotenogenic enzyme activities in the chloroplast, and (d) immunologically unrelated proteins are present in green and ripening fruit, which cross-react with anti-phytoene synthase sera.

The Accumulation of Carotenoids and Enzyme Activities

The large, characteristic (12-fold) increase in total carotenoids during fruit ripening (Fig. 1A) is primarily due to the accumulation of two carotenes, lycopene and β-carotene (Table I). The presence of lycopene and also ζ-carotene, phytofluene, and phytoene is unusual in plants since they are normally found only following treatment of the plant with bleaching herbicides (Bramley, 1993). The high amount of lycopene found in ripe fruit suggests that only a proportion of that formed in vivo can be cyclized to β-carotene in chromoplasts and may account for the accumulation of lycopene precursors in the pericarp of ripening fruit. The presence of lycopene cyclase in chromoplasts at higher levels of activity than the desaturase (Fig. 1B) lends support to the suggestion that separate compartmentation of the desaturase and cyclase enzymes occurs in the organelle, perhaps by means of two different chromoplast types (Laval-Martin et al., 1975). Alternatively, lycopene cyclization may be rate limiting in normal ripe fruit. It has been shown that tomato cultivars homozygous with respect to the *B* gene con-

tain high levels of β-carotene at the expense of lycopene (Premachandra, 1986).

The accumulation of carotenes in ripening fruit is accompanied by, or possibly preceded immediately by, a general decrease in carotenogenic enzyme activities (Fig. 1B). An explanation for the accumulation of carotenoids, despite the reduced amounts of enzyme activities, is that since the green fruit are photosynthetically active (Piechulla et al., 1987) the carotenoids are required as photoprotectants and hence will be turned over rapidly, thus necessitating high levels of carotenogenic enzyme activity. As the fruit ripen photosynthetic activity decreases and the Chl disappears (Fig. 1A); therefore, the carotenoids are not degraded as quickly. In addition, there will be some metabolism of xanthophylls into ABA in green fruit (Scolnik, 1987). Since carotenes rather than xanthophylls accumulate in ripening fruit, the enzymes catalyzing the oxidation steps to form the latter must be either absent or inhibited in chromoplasts.

Considering ripening fruit alone, the phytoene synthase activity is the only one of the three carotenogenic enzymes assayed that increases at any stage of ripening. This is paralleled by an increase in *Psy* mRNA, suggesting that the synthase, encoded by *Psy*, is the pacemaker enzyme in carotenoid synthesis in ripening fruit and that, at least in part, it is regulated at the level of transcription. The use of IDP as the substrate for phytoene synthase assays was found to be necessary, since the tomato stroma did not utilize FDP or GGDP, confirming reports that the tomato fruit contains a functional complex of the enzymes from IDP isomerase to phytoene synthase, which will not utilize exogenous FDP or GGDP (Maudinas et al., 1975, 1977).

The products of phytoene desaturase in vitro are different in green and ripening fruit (Table II). On initiation of ripening there is a large increase in the amount of lycopene produced rather than the formation of phytofluene and ζ-carotene. This coincides with the accumulation of lycopene in the tissue. One explanation for this phenomenon is that catalytic activity of chromoplasts favors full desaturation, whereas that in chloroplasts does not. Since there are two desaturases responsible for the conversion of phytoene into lycopene, the second of which converts ζ-carotene into lycopene (the "ζ-carotene desaturase"; Bramley, 1993), then this enzyme may be induced in high amounts at the breaker stage. The enzyme encoded by the *Pds* gene, which is expressed maximally at the breaker stage, is the phytoene desaturase (Pecker et al., 1992a), i.e. forms ζ-carotene from phytoene, and therefore cannot be exclusively responsible for lycopene production. Isolation of the ζ-carotene desaturase gene (Linden et al., 1993b) will enable its transcription to be estimated during fruit development. It is noticeable that the desaturase products of flower chromoplasts also favor lycopene production (Table II). The increase in phytoene synthase activity at 7 d.p.b. presumably explains the accumulation of phytoene (and probably phytoene epoxide) in 7- and 14-d.p.b. fruit (Table I), which had also been detected in an earlier study on ripe fruit (Mackinney et al., 1956).

The *Psy1* gene product has been shown to be targeted to the chloroplast, where it forms a labile association with the plastid membrane (Bartley et al., 1992; Lawrence et al., 1993). Our data concerning the active enzyme show that it is present in the stroma of both chloroplasts and chromoplasts, with only a very small amount associated with membranes (Table III). This result is in agreement with the stromal location of phytoene synthase in chloroplasts, etioplasts, and amyloplasts of *Capsicum annuum*, *Pisum sativum*, and *Hordeum vulgare* (Dogbo et al., 1987).

Gene Expression in Leaf and Fruit

The in vitro activities of phytoene synthase and desaturase in fruit during development and ripening (Fig. 1B) are not paralleled by corresponding changes in *Psy* and *Pds* transcripts. Maximum levels of steady-state mRNA are found in breaker fruit, whereas the highest enzyme activities are in green tissue. The absence of detectable mRNA in leaf and green fruit tissues by northern blot analysis, despite the presence of high enzymic activities in chloroplasts, supports the concept suggested by studies of transgenic plants containing antisense RNA to phytoene synthase (Bird et al., 1991) that there are at least two synthase and possibly two desaturase genes in the tomato. Although two independent genomic regions (GTOM5 and clone F) have been shown to hybridize to pTOM5 cDNA (Ray et al., 1992), this study provided no evidence of expression of either pTOM5-related gene in green tissue. It has been shown recently that *Psy1* mRNA can be detected in green tissues by an amplification procedure using reverse transcriptase PCR, but the level is 25-fold less than that in breaker fruit; therefore, it is unlikely to account for the high enzyme activities of green fruit (Giuliano et al., 1993).

The *Psy* and *Pds* clones therefore represent the genes expressed primarily in ripening fruit and in flower, i.e. chromoplast-containing cells, whereas other genes for each enzyme are presumably expressed in green, chloroplastidic tissue and may also be expressed in ripe fruit, although at a lower level. Recent studies of the genetic complementation of a phytoene synthase-deficient mutant of tomato suggest that three phytoene synthase genes are expressed in the plant: one in green tissue, another in ripening fruit, and a third in the flower (Fray and Grierson, 1993). Multigene families have been found for other steps in the isoprenoid pathway, e.g. hydroxymethylglutaryl-CoA reductase (Narita and Gruissem, 1989; Chye et al., 1991; Park et al., 1992).

Further experimental support for developmental regulation of the expression of phytoene synthase genes comes from the immunological properties of the proteins in green and ripe fruit. The *Synechococcus* anti-phytoene synthase serum cross-reacts with phytoene synthase in green tissue only, both on western blots (Fig. 3) and by immunoprecipitation of enzymic activity (Fig. 5). In contrast, the monoclonal antibody only cross-reacts with phytoene synthase from ripe fruit stroma (Fig. 4). The proteins in the two types of tissue are also of different molecular masses. It has not been possible to obtain similar immunological evidence for two *Pds* genes because of the lack of suitable, specific antibodies, but the change in phytoene desaturation products at the stage of breaker fruit (Table II) indicates a possible difference in the properties of the desaturase enzyme.

It has been suggested that only one form of phytoene desaturase is present in all tissues, based upon sequence

homology of the tomato (fruit) and soybean (cotyledon) enzymes (Pecker et al., 1992a). It has also been claimed that this enzyme is rate limiting in carotenoid biosynthesis in tomato (Pecker et al., 1992b) but not in *Capsicum*, where the GGDP synthase is reported to be rate limiting with phytoene desaturase showing no significant increase in enzymic activity or mRNA levels during fruit ripening (Hugueney et al., 1992; Kuntz et al., 1992). Our data suggest that the fruit-ripening-enhanced *Psy* gene product catalyzes the rate-limiting step in the carotenoid pathway of ripening tomato fruit and that the enzymes in green tissue are different from those encoded by *Psy* and *Pds*. In ripening fruit the transcription of these genes may control carotenoid formation, but at present this cannot be ascertained for chloroplastidic cells. Studies are underway to elucidate this possibility.

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