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The regulation and genetic manipulation of carotenoid biosynthesis in tomato fruit

Peter M Bramley

Division of Biochemistry, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK

Abstract: The Ailsa Craig variety of tomato (*Lycopersicon esculentum*) has been transformed with carotenoid genes from higher plants and bacteria. Progeny have been analysed for their carotenoid levels, carotenogenic enzyme activities and levels of gene expression. Ultrastructural studies have revealed changes in plastid structure. A similar approach has also been adopted with the high pigment (*hp*) mutant variety, which has elevated levels of carotenoids compared with the parental cultivar.

INTRODUCTION

The ripening of tomato (*Lycopersicon esculentum*) fruit is a highly regulated process during which the colour, flavour, 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 (ref. 1). The change in pigmentation is due to a massive accumulation of lycopene within the plastids and the disappearance of chlorophyll. The chloroplasts of mature green fruit change into chromoplasts, which contain lycopene in membrane-bound crystals (ref. 2). Early biochemical studies on tomato varieties were the basis on which the desaturase pathway from phytoene to lycopene was established (ref. 3). The carotenogenic enzymes of higher plants are located within the plastid (ref. 4), but their encoding genes are nuclear (ref. 5).

Analysis of tomato fruit, at 5 stages of development and ripening, has shown that the greatest carotenogenic activities are in green fruit, with phytoene synthase being located in the plastid stroma, whereas phytoene metabolism is associated with the plastid membranes (ref. 6).

In order to modify carotenoid levels in the tomato and to understand the regulatory mechanisms that control the formation and deposition of carotenoids in tomato fruit, we have used two approaches. Firstly, a study of the high pigment (*hp*) mutant of tomato which has elevated levels of carotenoids (ref. 7) and, secondly, production of transgenic varieties which overexpress either the tomato phytoene synthase (*Psy*) cDNA (ref. 8), or the *crtI* (phytoene desaturase) gene from *Erwinia uredovora* (ref. 9).

Tomato has been chosen for several reasons. Firstly, the several-hundred-fold increase in carotenoids during ripening and the rapid transition from mature green to red ripe fruit facilitates measurements of pigment levels and gene expression. Secondly, tomato is easily modified by genetic techniques. Thirdly, it is a very important food crop with major use in the processing and the fresh market sectors.

CHARACTERISATION OF THE HIGH PIGMENT MUTANT

Analysis of the pigments in *hp*, in comparison to the Ailsa Craig parental strain, showed that the pericarp of both green and ripe fruit of the former contained increased levels of carotenoids (Table 1), showing that the mutation affects both chloroplast-containing and chromoplast-containing fruit.

TABLE 1. Carotenoids levels in pericarp of Ailsa Craig and *hp* mutant varieties

Variety	Carotenoid content (μg/g fresh weight)	
	Green	Ripe
Ailsa Craig	18.2	173
hp	29.1	275

TABLE 2. Major carotenoids of ripe fruit pericarp of Ailsa Craig and *hp* varieties

Variety	Carotenoid (% total)					
	Xanth	Lyc	β-C	δ-/γ-C	PF	P
Ailsa Craig	5.0	60.1	20.2	2.9	1.7	8.0
hp	5.2	59.8	20.2	2.8	1.1	8.1

δ-/γ-C, δ- and γ-carotenes; β-C, β-carotene; PF phytofluene; P, phytoene; Lyc, lycopene; Xanth, xanthophylls

Further analysis of the pericarp carotenoids of ripe fruit by HPLC revealed a very similar pattern of individual carotenoids in each cultivar (Table 2), indicating that the mutation is within a regulatory gene.

Measurement of phytoene synthase and desaturase enzyme activities *in vitro*, using standard protocols (ref.6) revealed little difference, on a specific activity basis, between green and ripe fruit. Therefore the increased levels of carotenoids (Table 1) cannot be accounted for by higher levels of carotenogenic enzymes.

Light microscopy of the ripe fruit pericarp, however, revealed a key difference between the two varieties. Although the size of chromoplasts was the same in each variety, the number of plastids per cell was some two-fold more in the *hp* mutant (1140 in *hp*, 540 in Ailsa Craig).

In conclusion, therefore, the mutation in *hp* that causes an increase in carotenoid levels in ripe fruit has affected the number of plastids per cell, thus increasing the amount of carotenoid that can accumulate in the tissues.

MODIFICATION OF TOMATO CAROTENOID LEVELS BY TRANSFORMATION

Choice of genes vector, construction and transformation

Phytoene synthase (Psy)

Earlier studies had shown that the phytoene synthase gene is strongly expressed during fruit ripening, with an increase in enzyme activity at the breaker stage (ref. 6). Therefore, we ligated the *Psy* cDNA (ref. 8) into a pUC18-derived vector p JR1 to produce p JRex5 under the control of the CaMV 358 constitutive promoter. The vector was transferred to *Agrobacterium tumefaciens* LBA 4404 by triparental mating, tomato stem segments were transformed and transgenic lines selected on the basis of kanamycin resistance.

Phytoene desaturase (crt I)

The *crt I* gene of *Erwinia uredovora*, linked to the pea Rubisco small subunit transit sequence and under the control of the CaMV promoter had been used previously to transform tobacco, resulting in elevated carotenoid levels in leaves (ref. 10). We have used the same construct to transform tomato stem explants and have selected transformants, as described above.

Analysis of transgenic cultivars

Phytoene synthase transformants

The primary transformants produced a range of phenotypes. Some 40% produced yellow fruit, containing very low levels of carotenoids comparable to those in *Psy* antisense fruit (ref. 11).

This effect is probably due to co-suppression (gene silencing). Other transformants exhibited unscheduled carotenoid deposition in whole plant tissue as well as immature fruit (ref. 11). The consequence of this was the accumulation of lycopene in mature green fruit (Table 3). However, the final levels of lycopene in the red ripe fruit were lower than in control tissue, suggesting that premature deposition of lycopene limits its accumulation during ripening. Plastid number had not changed in the transformants, but in co-suppressed fruit the plastids were clustered around the nucleus. The reason for this is currently unknown.

TABLE 3. Overexpression of phytoene synthase: effect on carotenoids in green and ripe fruit

	Total	Lycopene
	μg/g fresh weight	
Control fruit		
Mature green	6.3	0
Ripe (14dpb)	126	65
Overexpressing line		
Mature green	14	1.6
Ripe (14dpb)	60	34

dpb, days post breaker

Phytoene desaturase (crt I) transformants

Primary transformants exhibited three different colour phenotypes: red, orange-red and orange. No unscheduled colouration was observed, nor any co-suppression effects. Analysis of total carotenoid levels and individual pigments in ripe fruit revealed that there was a modest change in total carotenoids compared to the untransformed control (372 and 315 μg/g fresh weight, respectively), but the profile had changed with an increase in β-carotene levels in transformants (Table 4).

The transgene was stably inherited and homozygous progeny maintained the same colour phenotype as primary transformants. The progeny were also resistant to the bleaching herbicide, norflurazon, as found for the transformants of tobacco (ref. 10).

In conclusion,constitutive expression of the *Erwinia crtI* gene caused an accumulation of β-carotene in ripe fruit, especially the columella. The reasons for this change are currently under investigation. Preliminary results with an antibody to the CrtI protein have shown that its level is higher in ripening fruit of the transformants than in the control fruit.

TABLE 4. Overexpression of *crt I*: effect on carotenoid levels in ripe fruit

	Total	Xanth	Lyc	β-C	γ-/δ-C	PF	P
	µg/g FW	(% total)					
Ailsa Craig							
Pericarp	183	1.5	76	10	tr	2.2	3.9
Columella	119	2.5	44	40	5.0	0.7	2.8
Jelly	13	8.9	17	68	0.3	tr	tr
Crt I transformant							
Pericarp	241	0.7	73	23	0.3	tr	tr
Columella	95	14	23	57	4.3	1.2	tr
Jelly	36	2.8	11	73	0.2	2.2	tr

See Table 2 for abbreviations

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