Running title: Elevation of β–carotene in tomato fruit by genetic modification

Genetic modification of tomato with lycopene β–cyclase gene produces high β–carotene and lycopene fruit

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Dedication

This work is dedicated to the memory of Prof Peter Böger, who kindly provided the opportunity for PDF and PMB to work in his laboratory on the mode of action of bleaching herbicides.

Abstract

Transgenic *Solanum lycopersicum* plants expressing an additional copy of lycopene β–cyclase (*LCYB*) from *Nicotiana tabacum*, under the control of the *Arabidopsis* polyubiquitin promoter (*UBQ3*), have been generated. Expression of *LCYB* was increased some 10-fold in ripening fruit compared to vegetative tissues. The ripe fruit showed an orange pigmentation, due to increased levels (up to 5-fold) of β–carotene, with negligible changes to other carotenoids, including lycopene. Phenotypic changes in carotenoids were found in vegetative tissues, but levels of biosynthetically related isoprenoids such as tocopherols, ubiquinone and plastoquinone were barely altered. Transformants showed tolerance to the bleaching herbicide β–cyclase inhibitor, 2-(4-chlorophenylthio) triethylamine. The phenotype was inherited for at least 3 generations.

Keywords: *Solanum lycopersicum*, lycopene β–cyclase, β-carotene, lycopene, genetic modification.

Introduction

Carotenoids are a major group of isoprenoids, widely distributed in nature [1]. Several of them are reported to have health benefits [2], most notably reduction in the incidence of prostate cancer from dietary lycopene [3] and alleviation of Vitamin A deficiency by β–carotene, which is pro-vitamin A [4]. Deficiency of Vitamin A causes xerophthalmia, blindness and premature death, especially in children aged 1-4 [5]. Since humans cannot synthesise carotenoids *de novo*, these health-promoting compounds must be taken in sufficient quantities in the diet. Consequently, increasing their levels in fruit and vegetables is beneficial to health. Tomato products are the most common source of dietary lycopene. Although ripe tomato fruit contain β-carotene, the amount is relatively low [1]. Therefore, approaches to elevate β–carotene levels, with no reduction in lycopene, are a goal of plant breeders. One strategy that has been employed to increase levels of health promoting carotenoids in fruits and vegetables for human and animal consumption is genetic modification [6].

Lycopene, an acyclic carotenoid, and precursor of β–carotene, is formed from the sequential desaturation of phytoene. These reactions are catalysed by the enzymes phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). The desaturase sequence occurs in the *cis* geometric isomer configuration and the action of a carotene isomerase (CRTISO) converts either *cis*-neurosporene or poly-*cis* lycopene to all-*trans* lycopene prior to cyclisation [7]. In green tissues the action of light and chlorophyll are believed to overcome the necessity for CRTISO activity [8]. Cyclisation of lycopene in tomato fruit yields β-carotene, via the action of two β-cyclase enzymes. LCYB is believed to predominate in the formation of vegetative carotenoids, whilst the *CYCB* gene shows ripening specific expression and is thus associated with β-carotene production during ripening [9]. In vegetative tissues, ε-carotene is formed via the action of a ε-ring cyclase and β-ring cyclase, leading to the synthesis of lutein, via hydroxylation, which is the predominant vegetative carotenoid [10]. The complete pathway is shown in Fig. 1.

Since the herbicide 2-(4-chlorophenylthio) triethylamine (CPTA) is known to inhibit β–carotene formation in tomato fruit [11], the transgenic lines have been grown in its presence to determine if overexpression of *LCYB* can overcome this inhibition.

Materials and Methods

Plant material

Tomato (*S. lycopersicum* cv Ailsa Craig) plants were grown in the glasshouse with supplementary lighting. Three plants per genotype were grown in a randomized manner concurrently with their respective backgrounds and fruit harvested at mature red ripe (7 days post breaker, dpb). Two or more fruits per plant were pooled to provide one replicate per plant and three per genotype.

Construction of the pVBLCYB vector

Digestion of pUC19 with *Sma1* released the *LCYB* cDNA as a 1.6 kb fragment, including the transit peptide, which was cloned into the *Sma1* site of the pSP vector (Promega), containing the polyubiquitin promoter (*UBQ3*; At5g03240; UniProt KB-Q1EC66) from *Arabidopsis thaliana,* the nopaline synthase terminator, the *nptII* gene for kanamycin resistance, controlled by the *AoPR1* promoter from *Asparagus officianalis*. The *Age1* fragment was sub-cloned into the pVB6 plant binary vector and designated pVB*LCYB* (Fig 2). The vector/insert was sequenced to confirm the manipulations. *E .coli* strain XL1-Blue (Promega) was used in these experiments. Sequences of the tobacco and tomato LCYB are shown in Supplementary Material, Fig 1.

Transformation of tomato plants

Agrobacterium tumifaciens LBA 4404 was transformed with pVB*LCYB* by triparental mating, using the helper plasmid pRK2103 [12]. Stem explants of tomato were transformed and regenerated as described previously [13]. Kanamycin (50 mg/ml) and carbenicillin (250 mg/ml) were used to select resistant transformants.

DNA and RNA analyses

DNA was extracted for PCR analysis from leaf material (ca. 200 mg), as described previously [13]. The presence of the transgene in the primary transformants (T_0) was confirmed by PCR using primers designed to amplify a 500 bp internal fragment of *LCYB*. Southern blot analysis of the T_0 and 1st generation (T_1) plants was carried out using the cetyltrimethylammonium bromide (CTAB) method [14]. DNA was digested with the appropriate restriction enzymes, fragments separated in 1% (w/v) agarose gels and transferred to Hybond N^+ (Amersham) overnight in 20% saline sodium citrate (SSC) solution. ^{32}P-Labelled fragments (using the Stratagene Random primer labelling kit, Prime It II), corresponding to the *LCYB* and *nptII* regions of the transgene, were used to probe the filters. Hybridisation, washing and detection were performed as described in the Hybond N+ instructions. Northern blot analysis was carried out on tomato fruit of the T_o and T_1 generations, harvested at 7 dpb [15]. Total RNA (20 mg) from the fruit was electrophoresed on 1.4% agarose/formaldehyde gels and then transferred to Hybond-N^+ in 20x SSC overnight. The 1.6 kb cDNA fragment was radiolabelled with 32P, as described above. Following pre-hybridisation, the filter was hybridised at 65^{o}C in 7.5% SDDS, 100mM phosphate buffer, pH 7.5. Each filter was washed for 15 min in 2X SSC, 0.5% SDS and then twice for 15 min with 1xSSC, 0.5% SDS and finally monitored for radioactivity for 24 h with a phosphorimager (MAcBAS V2.2, Fuji Photoimage Co.).

Treatment with CPTA

Sterilised seeds (ca. 30) from the homozygous T_1 line C4S1-15 and azygous control were germinated on Murashige and Skoog (MS) agar containing the herbicide 2-(4-chlorophenylthio) triethylamine (CPTA) at 30 and 40 µM. The seedlings were grown in a controlled environment with a 16 h photoperiod and day and night temperatures of 23 and 19^{o}C, respectively. They were assessed for germination and bleaching after 2 weeks and samples taken for isoprenoid analysis.

Analysis of isoprenoids

The isoprenoids of T_0, T_1 and T_2 progeny of leaf and 7 dpb fruit were extracted, separated, identified and quantified HPLC analyses, as described in [16].

Results

Plants expressing the *LCYB* gene from tobacco showed increased levels of β–carotene in ripe fruit and changes to leaf carotenoids

Following transformation, progeny were selected on the basis of kanamycin resistance (*nptII*) and PCR analysis. During regeneration, growth and development of vegetative tissues from all transgenic lines appeared phenotypically normal. However, as fruit ripening commenced, virtually all transgenic lines showed a greater degree of orange colouration in their fruit compared to the control. Analysis of the T_0 generation showed that increases in β-carotene content of the fruit were responsible for the altered colouration (Table 1). Of the T_o transformants, 85% exhibited a lycopene to β-carotene ratio within a range of 2:1 to 6:1, due to increases in β–carotene, with the wild type (Ailsa Craig) ratio being 7.46:1. Analysis of the T_1 and T_2 generations established that the phenotype was inherited (Table 1).

Insertions at a single locus were determined by Southern blotting using a *nptII* probe. Of the 20 transformants, 5 lines were single copy, while the others had copy numbers ranging from 2-4 for the *nptII* gene (data not shown). Homozygous lines were generated from single copy primary transformants, showing a range of β-carotene contents (603 C1S1, C1S2, C2S1 and C4S1, with lycopene: β-carotene ratios of 1.5, 2.99, 2.05 and 3.24, respectively; Table 1). Zygosity was assigned by Southern analysis using the *nptII* probe, co-segregation was assessed by expression of the transgene and inheritance of the phenotype by HPLC analysis. Co-segregation occurred in all cases and the high β-carotene phenotype was retained in the third generation (T_2). As shown in Supplementary Material, Fig. 2, the ratio of lycopene:β-carotene ratio differed between azygous, hemizygous and homozygous fruit. Although significant elevations in β-carotene were found in fruit of the transgenic lines, the content of biosynthetically related carotenoids such as phytoene, γ-carotene, δ-carotene, α-carotene, lutein, zeaxanthin, violaxanthin and neoxanthin was not altered. The

pattern of β-carotene and lycopene geometric isomers found in the transgenic fruit were identical to the wild type (i.e. β-carotene 90-95% all-*trans*, 10-5% *cis*; all-*trans* lycopene 96-98%, 15-*cis*- lycopene 1-4% and 9-*cis* lycopene 0.6%). Alterations in other fruit isoprenoids were minor, with increases of 0.35, 0.50 and 0.01-fold for tocopherols, plastoquinone and ubiquinone, respectively, in line 603 C4S1-15.

There were also changes to the carotenoid levels in transgenic leaves. For example, in the leaves of line 603 C4S1 all the carotenoids were increased, with lutein exhibiting the largest elevation (Table 2).

LCYB gene expression correlated with increased β–carotene levels in fruit

Northern blot analysis showed a positive correlation between the level of transgene expression and increased β-carotene formation (Fig. 3). A comparison of expression between chloroplast-containing (leaf and green fruit) tissues and ripe fruit showed that although the *UBQ3* promotor was constitutive in nature, expression was approximately 10-fold greater in ripe fruit (data not shown).

Transgenic lines showed tolerance to CPTA

A comparison of Ailsa Craig seedling and those of the transgenic line 603 C4S1-15, in the presence of CPTA, showed dramatic differences in plant vigour. The control seedlings showed significant chlorosis, whilst those of the transformed line remained green (Supplementary Fig. 3). Pigment analysis of the leaves revealed lower levels of carotenoids in the control (55% reduction at 40 µM CPTA), and qualitatively, β–carotene levels were

some 4.3–fold less in the control leaves (Table 3). Increases in lycopene in these leaves were also observed. There was also an increase in lutein levels in 603 C4S1-15 in comparison to Ailsa Craig at both CPTA concentrations.

Discussion

There have been several reports of using genetic modification for targeted and untargeted increases in carotenoid levels in crop plants, albeit with varying degrees of success [17]. With respect to β-carotene, a modest, 1.5-fold, increase was achieved using the *PSY-1* gene with a constitutive promoter (CaMV 35S) [18], whist transformation with the *Arabidopsis LCYB* and *PDS* promoter elevated β-carotene some 7-fold [19]. Alternatively, a bacterial phytoene desaturase, *CrtI* resulted in a 3-fold increase, but a decrease in lycopene content [20]. This was surprising, as CRTI catalyses lycopene, not β–carotene biosynthesis. A similar result was found using *CrtI* in Golden Rice [21] and may be caused by a feed-forward mechanism for the upregulation of *LCY* or *CYC-B*. Therefore, although increases in β-carotene were found, unintended perturbations to other carotenoids and related isoprenoids occurred, or else the total carotenoid content was reduced.

In contrast, in the present study levels of β–carotene have been increased up to 6-fold, without a decrease in lycopene, perturbations to other fruit carotenoids, nor to the detriment of plant vigour. The phenotype was stable for at least 3 generations (Table 1). Therefore, the transgenic fruit contain an improved carotenoid profile compared with non-transgenic cultivars and introgression lines [22]. This is the first report of using the *UBQ3* promoter in tomato, although it has been used with other dicot plants [23]. Although constitutive, the *UBQ* promoter gave differential expression levels of the transgene in vegetative and fruit tissues, with lower expression found in chloroplast-containing tissues that probably prevented the occurrence of gene silencing and/or co-suppression of the endogenous gene [24], since the homology of the tobacco and tomato β–cyclases is 87.2% (Supplemental Material, Fig 1). Since the endogenous *LCYB* gene is down regulated during fruit ripening [9], co-suppression is not an issue. Despite the low expression in vegetative

tissue, changes to carotenoids do occur (Table 2), most specifically a 4.4-fold increase in lutein, suggesting that LCYB catalyses the rate limiting step in the formation of this xanthophyll.

Growth of the transgenic lines with CPTA did not cause changes to plant vigour, unlike the chlorosis shown with the wild type (Supplementary Material, Fig. 3). Lycopene did not accumulate in the *LCYB* line (Table 3), indicating that expression of the *LCYB* transgene had caused tolerance to CPTA due to increased activity of LCYB. Therefore, such an approach is feasible for the production of a crop resistant to this herbicide and for screening putative cyclase inhibitors that have different modes of action to CPTA [25]. The increase in total carotenoids in the transgenic line is consistent with the report that CPTA modulates mRNA levels of carotenoid genes [26].

Nutritionally, an average ripe *LCYB* transgenic fruit contains virtually all the RDA for vitamin A compared to the wild type, which has about 39% of the RDA [27]. For comparison, the *crtI* transgenic tomato contains similar levels of β-carotene (ca. 70% RDA), but only 50% of the wild type lycopene content. Thus, this new genotype not only has a significantly elevated provitamin A content, but maintains its level of lycopene, compared to the wild type.

In summary expression of *LCYB* under the control of the *UBQ3* promotor has resulted in the specific elevation of β-carotene in ripe tomato fruit, but no changes to other fruit carotenoids. There is much current debate concerning the acceptance of genetically modified plants. The present study has produced a tomato with the potential to reduce vitamin A deficiency and contribute towards a high antioxidant diet. Such germplasm will provide a valuable resource for further detailed analysis [28] and add to the genetic resources for nutritional benefit.

Acknowledgements

We are grateful to Dr Susanne Römer for providing the *NtLCYB* cDNA clone. The work was supported by the European Community FAIR Programme (# CT9616333). Syngenta Agrochemicals is gratefully acknowledge for the provision of glasshouse space and some

laboratory facilities. We thank Karen Bacon and Carol Fisher for maintenance of the tomato plants and Mark Harker for some of the Southern analyses.

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Table 1. Carotenoid contents of transgenic plants expressing an additional β-lycopene cyclase gene.

Plant line	Generation/ Zygosity	Carotenoid content (μg/g DW)			
		Lycopene	β-Carotene	Total[a]	Ratio L:β-C
Wild type	-	2133 ± 479	290 ± 29	2560 ± 508	7.46
603 C4S1	T_0	5546 ± 1397	1711± 103	7394 ± 1490	3.24
603 C1S1	T_0	1192 ± 370	783 ± 46	2112 ± 416	1.52
603 C2S1	T_0	2560 ± 297	1247 ± 37	3944 ± 334	2.05
603 C1S2	T_0	2773 ± 471	928 ± 81	3838 ± 552	2.99
Azygous control[b]	T_1	2009 ± 134	329 ± 53	2669 ± 204	6.11
603 C4S1-1	T_1, He	2240 ± 99	609 ± 82	2991 ± 181	3.68
603 C4S1-15	T_1, Ho	2069 ± 40	725 ± 28	2936 ± 32	2.85
603 C4S1-5	T_1, Ho	1493 ± 43	783 ± 53	2418 ± 96	1.91
603 C1S1-4	T_1, He	1493 ± 79	406 ± 6	2041 ± 85	3.68
603 C1S1-18	T_1, Ho	1770 ± 104	667 ± 59	2579 ± 163	2.65
603 C1S1-19	T_1, Ho	2560 ± 110	580 ± 16	3282 ± 126	4.41
603 C2S1-1	T_1, Ho	2133 ± 85	725 ± 36	3000 ± 122	2.94
603 C4S1-15	T_2, Ho	3250 ± 1455	623 ± 175	4034 ± 163	5.22

Fruit were harvested at 7 dpb. [a] Includes lutein, phytofluene, ζ-carotene and phytoene at 1.8, 0.3, 0.14 and 1.3 % of the total carotenoids, respectively. [b] represents the mean of azygous plants from 603 C4S1, C1S1 and C2S1 lines. He, hemizygous; Ho, homozygous; T_0, primary transformants; T_1, second generation; T_2, third generation. All values are the means of 3 replicates ± s.e.

Table 2. Carotenoid content of leaves of Ailsa Craig and *LCYB* transgenic lines

	Carotenoid (µg/g FW)					
Genotype	Total	β-Carotene	Lutein	Anthera	Viola	Neox
Ailsa Craig	83.7 ± 1.5	22.0 ± 0.36	33.7 ± 4.1	6.3 ± 1.5	13.4 ± 1.5	8.3 ± 0.04
% Total		26.3	40.3	7.53	16.0	9.90
603 C4S1	275.6 ± 0.73	33.7 ± 1.7	147.3 ± 0.40	21.4 ± 0.50	42.2 ± 0.13	31.0 ± 1.5
% Total		12.2	53.4	7.80	15.3	11.2

Values are the means of 3 replicates ± s.e. Anthera, antheraxanthin; Viola, violaxanthin, Neox, neoxanthin

Table 3. Carotenoid content of leaves from plants treated with CPTA

Plant id and treatment	Carotenoid content (µg/g FW)						
	Total	Lycopene	β-Carotene	α-Carotene	Lutein	Neox	Viola
AC + 30 µM CPTA	62.1	2.9 ± 0.1 (4.6)	4.7 ± 0.99 (7.5)	7.1 ± 0.5 (11.3)	44.7 ± 7.6 (72.0)	1.8 ± 0.3 (2.4)	0.9 ± 0.4 (1.4)
AC + 40 µM CPTA	47.4	1.4 ± 0.1 (3.0)	1.8 ± 0.2 (3.8)	7.3 ± 0.7 (15.6)	34.7 ± 0.1 (73.0)	0.4 ± 0.04 (2.1)	1.8 ± 0.2 (3.8)
603 C4S1-15 + 30 µM CPTA	76.9	0.6 ± 0.13 (0.8)	7.2 ± 1.5 (9.5)	6.1 ± 1.2 (8.0)	58.0 ± 6.1 (76.2)	2.2 ± 0.2 (2.8)	2.8 ± 0.2 (3.6)
603 C4S1-15 + 40 µM CPTA	106.2	0.6 ± 0.07 (0.6)	7.8 ± 0.7 (7.5)	3.3 ± 0.73 (3.1)	90.6 ± 6.9 (85)	0.6 ± 0.06 (0.6)	3.3 ± 0.13 (3.1)

Percentage of total carotenoids shown in brackets. Values are the means of 3 replicates ± s.e. Viola, violaxanthin, Neox, neoxanthin.

Figure legends

Fig1. Carotenoid biosynthesis in higher plants.

Abbreviations: GGPP, geranylgeranyl diphosphate; PSY-1 and -2, phytoene synthase 1 and 2; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCYE, lycopene ε-cyclase; LCYB, lycopene β–cyclase; CYC-B, fruit specific lycopene β–cyclase; CRTR-B1, β-carotene hydroxylase; CYP97A, P450 carotenoid β–ring hydroxylase; CYP97C, P450 carotenoid ε-hydroxylase; VDE-1, violaxanthin de-epoxidase; ZEP-1, zeaxanthin epoxidase; NXS, neoxanthin synthase.

Fig 2. Schematic diagram of the *LCYB* construct pVBLCY.

Abbreviations: *UBQ3*, ubiquitin 3 promotor; *LCYB*, *N. tabacum* lycopene β-cyclase; nptII, nopaline synthase terminator; ASe1, BamH1, Age1 restriction sites; LB, left border; RB, right border .

Fig 3. Northern blot analysis showing expression of the tobacco *LCYB* gene in transgenic lines.

A, primary (T_0) transformants: 1, control *Ailsa Craig* (1:7); 2, C1S1 (1:1.5); 3 C1S2 (1:3.0), 4,t C3S1 (not determined); 5, C4S1 (1:3.2); 6, C4S2 (1:5), 7, C5S1 (1:6.5).

B, T_1 progeny: 1, Ailsa Craig control (1:7); 2 C4S1-1, hemizygous (1:3.7); 3 C4S1-5, homozygous (1:1.9); 4, C4S1-8, homozygous (1:2); 5, C4S1-12, azygous (1:6); 6, C4S1-15, T_2 homozygous (1:5.2), 7, C4S1-18, azygous (1:8); 8, Control *Ailsa Craig* (1:7). The lycopene to β–carotene ratios are provided in parenthesis. Uniform RNA loading was verified by probing with actin.

Supplementary material

Fig. 1. Alignment of tobacco and tomato lycopene LCYB.

Aligned using EMBOSS WATER at
http://www.ebi.ac.uk/Tools/psa/emboss_water/index.html

Fig. 2. HPLC analysis of carotenoids present in ripe fruit from azygous, hemizygous and homozygous lines.

A, Homozygous *LCYB* transgenic line C4S1-5; B, hemizygous transgenic line C4S1-1, and C, azygous line C4S1-18. Illustrated are chromatograms recorded at 460nm.

Fig. 3. Ailsa Craig and transgenic plants grown with 30 µM CPTA

A, Ailsa Craig; B, T_1 homozygous line C4S1-15, both grown for 14 days