The kiwifruit lycopene beta-cyclase plays a significant role in carotenoid accumulation in fruit

Charles Ampomah-Dwamena1*, Tony McGhie2, Reginald Wibisono1, Mirco Montefiori1, Roger P. Hellens1 and Andrew C. Allan1

1 The New Zealand Institute for Plant and Food Research Limited, Private Bag 92 169, Auckland 1142, New Zealand
2 The New Zealand Institute for Plant and Food Research Limited, Private Bag 11 030, Palmerston North, New Zealand

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Abstract

The composition of carotenoids, along with anthocyanins and chlorophyll, accounts for the distinctive range of colour found in the Actinidia (kiwifruit) species. Lutein and beta-carotene are the most abundant carotenoids found during fruit development, with beta-carotene concentration increasing rapidly during fruit maturation and ripening. In addition, the accumulation of beta-carotene and lutein is influenced by the temperature at which harvested fruit are stored. Expression analysis of carotenoid biosynthetic genes among different genotypes and fruit developmental stages identified Actinidia lycopene beta-cyclase (LCY-β) as the gene whose expression pattern appeared to be associated with both total carotenoid and beta-carotene accumulation. Phytoene desaturase (PDS) expression was the least variable among the different genotypes, while zeta carotene desaturase (ZDS), beta-carotene hydroxylase (CRH-β), and epsilon carotene hydroxylase (CRH-e) showed some variation in gene expression. The LCY-β gene was functionally tested in bacteria and shown to convert lycopene and delta-carotene to beta-carotene and alpha-carotene respectively. This indicates that the accumulation of beta-carotene, the major carotenoid in these kiwifruit species, appears to be controlled by the level of expression of LCY-β gene.

Key words: Beta-carotene, carotenoid biosynthesis, gene expression, kiwifruit, lycopene beta-cyclase.

Introduction

Plants accumulate a huge variety of secondary metabolites. Carotenoids are one such group of compounds that are synthesized in the plastids, mainly the chloroplasts and chromoplasts, by enzymes that are nuclear-encoded (Hirschberg, 2001). Carotenoids are 40 carbon isoprenoids that contain polyene chains containing conjugated double bonds. These compounds are important in nature as they are involved in light-harvesting, photoprotection, and pollinator attraction in plants (Tracewell et al., 2001; Szabo et al., 2005; Dong et al., 2007). They can accumulate to give attractive yellow, orange, and red pigmentation to some flowers and fruit (Suzuki et al., 2007; Tanaka et al., 2008). They are also precursors of vitamin A, implicated in reducing the progression of diseases such as age-related macular degeneration, certain types of cancers, and cardiovascular diseases (Al-Babili et al., 2001; Paine et al., 2005; DellaPenna and Pogson, 2006; Aluru et al., 2008). Fruits that accumulate high levels of carotenoids therefore can potentially provide a rich source of these healthy compounds.

The carotenoid biosynthetic pathway in plants is shown in Fig. 1 (Cunningham et al., 1994; Hirschberg, 2001). The first committed step is the condensation of two molecules of geranyl geranyl pyrophosphate (GGPP) to form phytoene, catalysed by the enzyme phytoene synthase (PSY). The carotenoid pathway branches at the cyclization of lycopene, which is acted upon by two enzymes: lycopene beta-cyclase (LCY-β) and zeta-carotene desaturase (ZDS). These enzymes are responsible for the formation of beta-carotene and alpha-carotene, respectively. The expression of these genes is regulated by various environmental factors such as temperature, light intensity, and nutrient availability. The exact regulation of these genes is still not fully understood, but studies have shown that their expression is influenced by the environment in which the fruits are grown.

* To whom correspondence should be addressed: E-mail: cdwamena@hortresearch.co.nz
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Carotenoid biosynthetic pathway in plants. Enzymatic conversions are shown by arrows with the enzymes responsible in bold. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zeta-carotene desaturase; CRTISO, carotene isomerase; LCY-β, lycopene beta-cyclase; LCY-ε, lycopene epsilon-cyclase; CRH-β, beta-carotene hydroxylase; CRH-ε, epsilon-carotene hydroxylase.

Fig. 1. Carotenoid biosynthetic pathway in plants. Enzymatic conversions are shown by arrows with the enzymes responsible in bold. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zeta-carotene desaturase; CRTISO, carotene isomerase; LCY-β, lycopene beta-cyclase; LCY-ε, lycopene epsilon-cyclase; CRH-β, beta-carotene hydroxylase; CRH-ε, epsilon-carotene hydroxylase.

by lycopene cyclases to produce alpha-carotene and beta-carotene. The formation of alpha-carotene requires the addition of an epsilon ring to one end of the linear lycopene molecule (yielding delta-carotene) by the enzyme lycopene epsilon cyclase (LCY-ε), followed by the activity of lycopene beta-cyclase (LCY-β), which adds a beta ring to the other end of the chain. In contrast, beta-carotene results from the addition of two beta rings to both ends of the linear lycopene molecule by the lycopene beta-cyclase enzyme (LCY-β). The flux through the branches is thus dependent on the relative activities of the cyclases involved. In maize, polymorphisms in LCY-ε were found to account for the accumulation of beta-carotene and xanthophylls derived from this carotene (Harjes et al., 2008). Similarly, in the Arabidopsis lut1 and lut2 mutants, in which lutein accumulation is reduced or completely absent, there is an increased accumulation of beta-ring-containing carotenoid compounds (Pogson et al., 1996; Tian et al., 2003). Overexpression of beta-carotene hydroxylase in transgenic Arabidopsis increased the concentrations of xanthophylls without any significant reduction in the amount of other carotenoids, suggesting genetic manipulation of a single step can increase flux through the pathway (Davison et al., 2002).

The introduction of single biosynthetic genes into unrelated plant species influences the flux through the whole carotenoid pathway, indicating the pathway is conserved and enzyme activity maintained between species. The introduction of the Narcissus (daffodil) phytoene synthase (PSY), lycopene cyclase (LCY), and bacteria CRTI genes into rice produced a beta-carotene-rich variety (Ye et al., 2000; Beyer et al., 2002). Similarly, the co-expression of the daffodil PSY and bacteria CRTI genes increased beta-carotene rather than lycopene in rice endosperm (Ye et al., 2000; Al-Babili et al., 2006). This suggests that the carotenoid pathway in rice is limited by the enzyme steps converting phytoene to lycopene that were complemented by the introduction of heterologous genes through transformation.

Actinidia spp. (kiwifruit) show a considerable variation in fruit colour. The common commercial species are green-fleshed Actinidia delicosa and yellow-fleshed Actinidia chinensis. However, there are other species currently unavailable commercially that accumulate extremes of anthocyanin and carotenoid pigments (Fig. 2). As some green-fleshed Actinidia genotypes ripen, chlorophyll is degraded in the skin and flesh to reveal different pigmentation contributed by carotenoids and anthocyanins (Montefiori et al., 2005). There is little known about the distribution of carotenoid accumulation in kiwifruit or the alleles responsible for the inheritance of these potentially desirable variations in appearance. Such knowledge would be valuable to plant breeders, given the challenges of developing new kiwifruit varieties in a genus that is both dioecious and exhibits extensive variation in ploidy.

In order to understand the molecular basis of the diversity of carotenoid accumulation in kiwifruit, the levels of key carotenoid compounds have been measured across various genotypes with different pigmentation phenotypes. In addition, candidate genes that code for key steps in this biochemical pathway were identified in kiwifruit and expression patterns analysed. Our results suggest that the major carotenoids that accumulate in kiwifruit are beta-carotene and lutein and that the levels of beta-carotene are controlled by the transcriptional activity of the lycopene beta-cyclase gene.

Materials and methods

Plant materials

Kiwifruit varieties were selected from mapping and breeding populations available in New Zealand. Genotypes were chosen based on colour and collected over the fruit development phase. MP161, MP165, and MP214 were selected from an Actinidia chinensis mapping population (Cheng et al., 2004; Fraser et al., 2004) based on the colour of ripe fruit. MaMe1, 2, and 3 were collected from a cross between A. macrosperma and A. melanandra. Fruits were harvested at the mature green stage and stored at room temperature where samples were selected at different time points as skin colour changed during ripening. A. macrosperma fruit were harvested at a mature green stage for ripening at two different temperatures (4 °C and 20 °C). Fruit samples were picked at intervals and analysed for colour, gene expression, and carotenoid analysis.
Colour measurement

Fruit colour was measured at 2 cm below the skin surface on 10 individual fruit from each sample, using a Minolta Chroma Meter (C-100, Minolta Camera Co. Ltd., Osaka, Japan). The Chroma Meter was calibrated with a white tile and black card initially and periodically throughout analysis. The Chroma meter allocates colour coordinates to each sample using the 3-dimensional $L^3a^3b^3$ colour space (Humphries et al., 2004). The readings were calculated as Lightness ($L$), Chroma ($C$), and Hue angles ($H$) for each individual fruit and an average was taken for each sample to enable comparisons.

Nucleic acid isolation

Fruit samples were snap-frozen in liquid nitrogen and were kept at –80 °C or used immediately for RNA isolation. Total RNA was isolated from fruit tissue after homogenization using CTAB buffer (Chang et al., 1993). Homogenized tissue (1 g) was put into CTAB buffer at 65 °C and extracted twice with chloroform isoamylalcohol. The RNA was precipitated with 3 M LiCl overnight at 4 °C. The nucleic acid pellet was dissolved in SSTE buffer and extracted once with chloroform followed by precipitation with 2 volumes of absolute ethanol at –20 °C for 30 min. The RNA pellet was redissolved and further DNase treated to remove DNA contaminants. Genomic DNA was isolated from tissues using Qiagen plant DNA isolation kit following manufacturer’s instructions.

cDNA synthesis

cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Total RNA (0.5–1 μg) from each sample was used in a reaction with 50 μM oligo dT(12) primer, 500 μM dNTPs, 1× reverse transcription buffer, 5 mM MgCl$_2$, 10 mM DTT, 40 units of RNaseOUT™ and 200 units of Superscript III reverse transcriptase all in a total volume of 20 μl. The reaction was incubated at 50 °C for 50 min and terminated at 85 °C for 5 min.

Degenerate PCR cloning and quantitative PCR

Actinidia PSY was not found in the EST libraries previously published (Crowhurst et al., 2008). Degenerate PCR primer pairs: TATGTKGCTGGTACNGTT, and CTTGCATCTTCKCCAACN, spanning a moderately conserved region of aligned PSY sequences, were designed to amplify a 150 bp fragment from kiwifruit cDNA templates. This was followed by a nested RACE PCR using sequence specific forward primers F1 CATTGGGGGCTTTGTTGTGT and F2 TGCAGTTCCGGGACCTTAAGAAACTC in combination with GeneRacer (Invitrogen) primers 3′ P
GCTGCAAGGATAACCCTGTAACCG and 3’ NP CGCTACGTAAACGGCAGTACGTG, respectively.

Primers for *Actinidia* carotenoid biosynthetic genes were designed across predicted intron positions of each gene with Trimot of ~60 °C. Primer pairs used for amplification were as follows: Actin (TGCATGAGCGATCAAGTTTCAAG, TGTCATGAGCTGTTAGTAC), PSY (CGATGTTGAAAGGCAAGCAGAC, GTTCTCGAGGGGCAACATAG), PDS (AGCCAGAAGGGCCTCTCTGAGT, TCCCTCAGGTTGCAAAAACCA), ZDS (TGATGGTTTGGCACCACAAACAG, TGATCCCCACCTGAGATGAAA), CRTISO (GGACACCAAAGACTGCATTGTTTGCCACCAAAACAG, TGCAATCCCATCCCAGTG, TCCTCTGCAGGTGCAAAAACCA), ZDS (CAACAATAG) PDS (AGCAGAAGCCCCCTCTCAGGATTGAAGCCAACGACTAC, GTTCTCGAAGGGGCAACATAG).

Quantitative PCR analysis was performed using the LightCycler System (Roche LightCycler 1.5; Roche Diagnostics). All reactions were performed using the LightCycler FastStart SYBR Green Master Mix (Roche Diagnostics) following the procedure described by the manufacturer. Samples were prepared in three replicates. A negative control using water as template was included in each reaction. Data were analysed using the Lightcycler software version 4 and normalized to kiwifruit actin gene expression because of its consistency across fruit development. PCR efficiency was calculated for each gene using a standard curve of serial dilutions and used in relative expression analysis. For each group of samples, one was selected as a calibrator and assigned a nominal value of 1.0. Error bars indicate standard error of the mean of the technical replicates. The genes examined in this study were deposited in the GenBank database with the following accession numbers: ACTIN (FG454048), PSY (FJ797304), PDS (FG496959), ZDS (FG486987), CRTISO (FG435001), LCY-β (FG437038), LCY-ε (FG527328), CRH-β (FG482821), and CRH-ε (FJ797305).

Protein purification and enzyme assays

PCR-amplified cDNA of the lycopene beta-cyclase open reading frame, without a stop codon, was cloned between the NdeI and KpnI cloning sites of the pET30 vector (Novagen) and sequenced to identify the correct clone. The cloned vector was transformed into BL21 bacteria competent cells for protein induction and purification. Freshly streaked cells were grown overnight in Luria-Bertani (LB) broth and inoculated into a 500 ml culture of auto-induction medium (ZYM-5052) and grown at 16 °C for 48–72 h until an OD600 of ~10. Cells were harvested by centrifugation and the pellet frozen at ~80 °C overnight. The pellet was thawed on ice and resuspended in His-Ind buffer with a protease inhibitor. Resuspended cells were lysed by two passes through an EmulsiFlex-C15 high-pressure homogenizer (Avestin) with a pressure setting between 15 000–20 000 psi. Cell debris was removed by centrifugation at 15 000 rpm for 30 min at 4 °C and the supernatant was filtered through a 0.45 μm filter. The sample was loaded onto a precharged and equilibrated 5 ml Hi Trap column (Pharmacia). The bound proteins were washed with 50 ml 1× His Trap binding buffer (15–35 mM imidazole) and eluted at 2 ml min−1 using 1× His Trap elution buffers providing a 0–500 mM imidazole gradient on FPLC. Fractions corresponding to eluted protein peaks (at 280 nm) were identified.

Protein (15–20 μg) was resolved by electrophoresis on NuPAGE Novex 4–12% BIS-TRIS gel (Invitrogen) followed by staining with Coomassie Brilliant Blue or electro-transferred to a nitrocellulose filter for Western analysis. His-tagged proteins were bound with mouse anti HIS6 antibody followed by mouse alkaline phosphatase treatment and colour detection with NBT/BCIP.

Carotenoid and chlorophyll extraction

Freshly streaked carotenoid producing bacteria (Cunningham and Gantt, 1998) were cultured overnight in LB broth with antibiotics and inoculated into a 500 ml LB in a 2.0 l flask and grown at 28 °C, in the dark with shaking at 225 rpm. Cells were induced by adding IPTG (1.6 mM) and FeSO₄.
after the first 24 h and grown for another 24 h. Cells were harvested by centrifugation and resuspended in 0.5 ml of distilled water, and 4.5 ml of 10% KOH and carotenoids extracted using 1:1 methanol:diethyl ether (v/v) followed by 100% DEE. NaCl (5 ml of 5 M) was added to the DEE extract and the organic phase removed. This was followed by two washes with water and the organic phase was recovered and dried by flushing with N₂.

Carotenoid and chlorophyll were extracted from fruit samples using the method described earlier by Montefiori et al. (2005). Fruit tissue (1–2 g) was freeze-dried and homogenized in 5 ml acetone with 0.1% butylated hydroxytoluene (BHT) in the presence of 100 mg of Na₂CO₃ and 500 mg of anhydrous Na₂SO₄. Homogenates were stored at 4 °C, in the dark overnight. The supernatant was extracted using 2 ml of diethyl ether and 8 ml of 10% (w/v) NaCl with centrifugation at 3000 g for 10 min. The combined ether phases were taken to dryness by flushing with N₂.

**HPLC analysis of pigments**

The dried carotenoid samples were dissolved in 700 μl of 0.8% BHT/acetone and the pigment concentrations determined by reversed-phase high performance liquid chromatography (HPLC). A Waters HPLC system comprising an Alliance Separations Module (W2690), photodiode array detector (Model 996), and a fluorescence detector (474) was used. Pigment separation was achieved using a YMC C30 Carotenoid HPLC column (250×4.6 mm) at a flow rate of 1 ml min⁻¹ and 5 μl sample injections. Solvent A was HPLC grade methanol and solvent B was HPLC grade dichloromethane. A binary gradient was used with starting conditions of 95% A and 5% B changing linearly to 10% B at 5 min, 20% B at 15 min, 40% B at 30 min, and finally 60% B at 53 min. The composition was held at 60% B until 42.5 min and then returned to starting conditions and held for 5 min before the next injection. Carotenoid composition was monitored at 455 nm, and chlorophylls with fluorescence (ex 440 nm, em 660 nm). All chromatographic data were processed using a Chromleon Chromatography Management System (Dionex Corporation). Chromatographic peaks were identified by comparison of retention times and UV/Vis spectra with authentic standards of alpha-carotene, lutein, violaxanthin, antheraxanthin, zeaxanthin, chlorophyll a, and chlorophyll b. Concentrations were calculated by interpolation using a standard curve prepared from authentic standards for alpha-carotene, lutein, chlorophyll a, and chlorophyll b; all other carotenoids were quantified as lutein equivalents.

**Sequence analysis**

Candidates of the kiwifruit carotenoid biosynthetic genes were identified from an expressed sequence tags (EST) database (Crowhurst et al., 2008) using TBLASTN with known carotenoid genes (cut-off value of e-20). The candidates were completely sequenced and translated for alignment and tree reconstruction. Tree reconstruction was done using the MEGA 3.1 package program (Kumar et al., 2004) and the reliability of tree reconstruction was

![Fig. 4. Accumulation of beta-carotene (A) and lutein (B) in Actinidia chinensis genotypes; MP161 (white bars), MP165 (hatched bars), and MP214 (black bars). Fruits were picked at developmental stages 20, 50, 90, and 150 d after full bloom and the pericarp analysed for their carotenoid concentration using HPLC. Error bars are standard errors of the mean of three technical samples.](https://academic.oup.com/jxb/article-abstract/60/13/3765/529882)

| Fruit genotypes | Mmp161 | Mmp165 | Mmp214 |
|-----------------|--------|--------|--------|
| Fruit stage (DAFB) | 50 | 90 | 150 | 50 | 90 | 150 | 50 | 90 | 150 |
| Total carotenoids | 2.00 | 2.35 | 1.55 | 0.63 | 0.84 | 0.67 | 1.94 | 0.96 | 0.84 |
| Chlorophyll a | 8.07 | 6.68 | 0.09 | 2.25 | 2.98 | 0.36 | 3.82 | 1.26 | 0.00 |
| Chlorophyll b | 4.29 | 4.90 | 0.00 | 1.98 | 2.83 | 0.91 | 3.72 | 1.79 | 0.00 |
estimated by calculating bootstrap confidence limits based on 1000 replicates. The GenBank accession numbers of the proteins, or translated products, used in the analysis are shown in the legend of Fig. 3.

**Results**

**Bioinformatics and sequence analysis of carotenoid genes**

EST sequences representing the kiwifruit carotenoid biosynthetic genes, *PDS*, *ZDS*, *CRTISO*, *LCY-β*, *LCY-ε*, *CRH-β*, and *CRH-ε* were identified from an *Actinidia* EST database (Crowhurst et al., 2008) by TBLASTN analysis using sequences of known function from other species. No EST sequence was identified with sufficient homology to the phytoene synthase gene, suggesting *PSY* is expressed at a low level in the tissues used to generate the EST libraries. A 1.59 kb kiwifruit *PSY* was therefore isolated, using degenerate and RACE PCR, from *Actinidia chinensis* cDNA templates. Candidate carotenoid biosynthetic genes were present in *Actinidia delicosa* and *Actinidia chinensis* fruit and vegetative bud libraries. The kiwifruit PDS protein sequence has 87% identity to both the *Arabidopsis* (AT4G14210) and capsicum (CAA48195) PDS. The ZDS candidate showed 80% amino acid identity to *Arabidopsis* (AT3G04870) and 83% to tomato ZDS (EF650012). Two non-redundant *LCY-β* sequences were identified in our EST databases representing two genes *AdLCY-β*1 and *AdLCY-β*2 with 88% similarity. Both sequences could be found in the same kiwifruit species and the primers designed were able to detect transcripts from both genes. *AdLCY-β*1 (FJ427508) showed 56% identity to *Arabidopsis* (AT5G52570) and 53% to carrot *LCY-β* (ABB52071) and was phylogenetically more closely related to the tomato chromoplast-specific *LCY-β* (AAG21133), than the other *LCY-β*s examined (Fig. 3). The kiwifruit CRH-β has 67% and 63% identity, respectively, to the *Arabidopsis* genes AT5G52570 and AT4G25700.

**Carotenoid accumulation and gene expression in Actinidia**

Carotenoid accumulation patterns were measured in a range of *Actinidia* genotypes and individuals from

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**Fig. 5.** Expression of carotenoid biosynthetic genes in three *Actinidia chinensis* genotypes MP161, MP165, and MP214. The genes were phytoene synthase (*PSY*), phytoene desaturase (*PDS*), zeta-carotene desaturase (*ZDS*), carotenoid isomerase (*CRTISO*), lycopene beta-cyclase (*LCY-β*), lycopene epsilon-cyclase (*LCY-ε*), beta-carotene hydroxylase (*CRH-β*) and epsilon-carotene hydroxylase (*CRH-ε*). Total RNA was isolated from fruit pericarp at same developmental stages as in Fig. 4. Gene expression was determined relative to actin transcript levels in the samples.
Table 2. Carotenoid contents (µg g⁻¹ fresh weight) in fruit of an Actinidia macrosperma×A. melanandra cross by HPLC

Carotenoid concentrations were measured in fruit of three individuals from an Actinidia macrosperma×Actinidia melanandra cross (MaMe1, MaMe2, and Mame3) at matured green-fruit stage (S0) and three different stages of fruit-colour change during ripening (S1–S3). Data shown are means of four replicates.

| Genotype | Stage | Alpha-carotene | Lutein | Beta-carotene | Zeaxanthin | Antheraxanthin | Violaxanthin | Neoxanthin | Total carotenoids |
|----------|-------|----------------|--------|---------------|------------|----------------|--------------|------------|------------------|
| MaMe1    | S0    | 0.30           | 6.80   | 4.03          | 0.11       | 0.12           | 1.83         | 0.70       | 16.65            |
|          | S1    | 0.05           | 2.38   | 1.00          | 0.00       | 0.00           | 0.36         | 0.22       | 4.57             |
|          | S2    | 0.01           | 1.86   | 1.17          | 0.02       | 0.00           | 0.34         | 0.11       | 4.12             |
|          | S3    | 0.08           | 6.20   | 7.07          | 0.52       | 0.00           | 0.56         | 0.13       | 16.34            |
| MaMe2    | S0    | 0.02           | 2.10   | 0.40          | 0.06       | 0.01           | 0.27         | 0.19       | 3.69             |
|          | S1    | 0.00           | 2.10   | 0.60          | 0.00       | 0.00           | 0.11         | 0.06       | 3.20             |
|          | S2    | 0.02           | 2.46   | 1.84          | 0.04       | 0.00           | 0.15         | 0.04       | 5.65             |
|          | S3    | 0.31           | 3.81   | 3.45          | 0.37       | 0.00           | 0.26         | 0.12       | 8.92             |
| MaMe3    | S0    | 0.28           | 7.97   | 4.93          | 0.16       | 0.09           | 1.80         | 0.67       | 19.80            |
|          | S1    | 0.03           | 3.34   | 1.97          | 0.00       | 0.00           | 0.46         | 0.21       | 7.25             |
|          | S2    | 0.04           | 2.62   | 2.41          | 0.00       | 0.00           | 0.16         | 0.14       | 6.41             |
|          | S3    | 0.22           | 1.50   | 6.11          | 0.12       | 0.00           | 0.10         | 0.02       | 8.76             |

specific crosses (Fig. 2). Fruit at different developmental stages and exposed to a range of temperature treatments were also examined. The relationship between carotenoid levels and gene expression was also studied by examining the relative expression of carotenoid biosynthetic genes, PSY, PDS, ZDS, CRTISO, LCY-b, LCY-e, CRH-b, and CRH-e in the kiwifruit genotypes using real-time PCR.

Three Actinidia chinensis individuals (MP161, MP165, and MP214), with different mature fruit colours, were selected and their carotenoid concentrations measured during the fruit development period. Fruit colour was measured as an indicator of pigment accumulation during fruit development using a Chroma Meter. MP161 (yellow-fleshed), MP165 (green-fleshed), and MP214 (pale orange-fleshed) fruit at 150 d after full bloom (DAFB) had average hue angles of 96°, 108°, and 98°, respectively (Fig. 2A, B, C).

Carotenoid concentrations in Actinidia chinensis were measured by HPLC. None of the well-known linear carotenoids such as phytoene and lycopene was detected in these varieties; however, the cyclized carotenoids and the xanthophylls were present (Fig. 4). Of the cyclized carotenes, beta-carotene was present at the highest concentrations while only traces of alpha-carotene could be detected. Similarly, of the common xanthophylls present, lutein concentration was highest in most of the samples analysed, while zeaxanthin was detected at low concentrations. These results suggested a reduced efficiency of beta-carotene conversion to zeaxanthin and an increased hydroxylation of alpha-carotene to lutein.

The beta-carotene concentration in the fruit increased with developmental stage. A higher concentration of beta-carotene was measured at 150 DAFB [1.12 µg g⁻¹ fresh weight (FW)] in MP161 fruit compared with 0.33 µg g⁻¹ FW at 50 DAFB (Fig. 4A). In contrast, lutein concentrations decreased with developmental stage, with higher concentrations in fruit at 50 DAFB and lower concentrations in mature fruit (Fig. 4B). MP161 accumulated the highest lutein concentrations among the three genotypes of A. chinensis, with concentrations of 1.16 µg g⁻¹ FW of lutein at 50 DAFB, which reduced to 0.4 µg g⁻¹ FW at 150 DAFB (Fig. 4). The concentrations of chlorophyll a and b also decreased with developmental stage of these fruits (Table 1). Overall, beta-carotene and lutein were the dominant carotenoids in these A. chinensis individuals but showed opposing patterns of accumulation during fruit development.

Gene expression in Actinidia chinensis

In A. chinensis, the relative expression of PDS, measured by real-time PCR, was highest in 20, 50, and 90 DAFB fruits of all three genotypes compared with 150 DAFB fruit (Fig. 5). The expression profile of ZDS was similar to that observed for PDS, for all three genotypes. In MP165, ZDS expression was reduced between the 20 DAFB and 50 DAFB fruit stages, but the expression level increased at 90 DAFB before being down-regulated at 150 DAFB. Overall, the expression of PDS and ZDS showed similar patterns for the three genotypes and suggested that the expression of these two genes may be co-ordinately regulated.

The levels of LCY-b transcripts increased in all genotypes until 90 DAFB. Subsequently, in MP161 and MP165, there was a decrease in transcript expression levels. For MP214, this increase was maintained until maturity. The relative expression of CRH-b among the three genotypes differed significantly at 20 DAFB, where MP165 showed high expression compared with the other two individuals. The expression of PSY was similar to CRH-b, highest at 20 DAFB but decreasing towards fruit maturity for all three individuals. At later stages, similar levels of expression were observed for all three cultivars. The expression of the carotenoid biosynthetic genes in A. chinensis showed similar patterns except for LCY-b, LCY-e, and CRH-e expressions, which differed significantly among the three genotypes especially at 150 DAFB.
To investigate carotenoid biosynthesis in kiwifruit further, the carotenoid contents of three individuals from a population of A. macrosperma × A. melanandra (MaMe1, MaMe2, and MaMe3) were measured. In this cross that segregates for colour and pigment accumulation, A. macrosperma which is a high carotenoid-accumulating variety (McGhie and Ainge, 2002) was the maternal parent, and the male parent was A. melanandra. The concentrations of individual carotenoids were determined at 20 DAFB (S0) and also when fruit were mature green (S1). During fruit ripening, both the fruit skin and flesh changed colour to lime green (MaMe1), red (MaMe2), and orange (MaMe3) (Fig. 2G, H, I); therefore, carotenoid concentrations were determined at two ripening stages, when colour change was obvious (S2), and when the colour change appeared complete (S3) (Table 2).

The carotenoid concentrations of these individuals were generally higher than those of the A. chinensis population (Table 1). Fruit at S0 had the highest carotenoid concentrations, which reduced at fruit maturity and increased again during ripening. The concentrations of beta-carotene were always higher than those of alpha-carotene in these Actinidia species at all the stages examined, while lutein was present in higher concentrations than zeaxanthin (Table 2). Beta-carotene concentration was 24% of total carotenoids in MaMe1 fruit at S0, and increased during ripening to 40% at the S3 stage. A similar trend was observed for MaMe2 and MaMe3 fruit, with beta-carotene in MaMe3 accumulating to approximately 70% of total carotenoids in S3 ripened fruit (Table 2). Lutein concentrations increased to approximately 40% of total carotenoids in MaMe1 and MaMe2 fruit at the S3 stage whereas zeaxanthin was barely detectable in these fruit. By contrast, in MaMe3 fruit, the proportion of lutein was reduced to 17% by the S3 stage. The beta-ring xanthophylls antheraxanthin, violaxanthin, and neoxanthin were detected at very low concentrations in all three A. macrosperma × A. melanandra individuals (Table 2).

The subcellular localization of carotenoid accumulation was examined using light microscopy. An abundance of orange-coloured chromoplasts was observed in the ripe fruit of MaMe3 and MaMe2 compared with MaMe1 fruit (Fig. 6).

**Gene expression in individuals from A. macrosperma × A. melanandra cross**

In A. macrosperma × A. melanandra, PSY, PDS, and LCY-ε expressions were highest at 20 DAFB (S0) in all three individuals but was significantly reduced over the three fruit ripening stages (S1–S3) (Fig. 7). ZDS expression was reduced between S0 and S1 and did not increase during ripening in both MaMe1 and MaMe2. By contrast, MaMe3 showed a high ZDS expression both at S0 and an increase in expression during fruit ripening (Fig. 7). CRTISO expression showed no increase during fruit ripening while LCY-β expression was low at S0 for all three genotypes but was elevated at S1, S2, and S3 for MaMe1 and MaMe3, although not for MaMe2. MaMe2, compared to the other two genotypes, was a low carotenoid variety. The expression of beta-carotene hydroxylase (CRH-β) was down-regulated between S0 and the S1–S3 stages for all three genotypes while CRH-ε was up-regulated in MaMe2 at the S3 stage. Overall, beta-carotene concentrations appeared to...
be controlled by the combined gene expression levels of ZDS and LCY-β in these kiwifruit genotypes.

**Carotenoid accumulation during ripening of Actinidia macrosperma**

Post-harvest accumulation of carotenoids in *A. macrosperma* was examined at two different temperatures (20 °C and 4 °C). Fruit ripening at 20 °C was associated with an increased accumulation of carotenoids, resulting in an orange-coloured fruit within 27 d. The ripening process was delayed in fruit kept at 4 °C until they were transferred to 20 °C (Fig. 8). The total carotenoids in the stored fruit increased from an average of 6.90±1.30 μg g⁻¹ FW at T₀, to 46.99±15.12 μg g⁻¹ FW at T₃ (27 d of storage) when fruit were stored at 20 °C. However, if the fruit were stored at 4 °C, total carotenoids only reached 8.30±1.12 μg g⁻¹ FW at T₃, after 38 d of storage. This result suggested that carotenoid accumulation was inhibited by low temperature during storage. This was confirmed when fruits initially stored at 4 °C for 20 d increased their carotenoid content by about 6-fold (5.86±0.38 to 30.64±14.29 μg g⁻¹ FW) when transferred to 20 °C for 18 d of storage.

Beta-carotene was the major compound accumulating in these fruit, during ripening and accounted for most of the increase in total carotenoids (Fig. 9). At T₀, beta-carotene was 37.2% (2.57±0.9 μg g⁻¹ FW) of all measured carotenoids but the proportion of beta-carotene increased to about 90% (42.82±15.49 μg g⁻¹ FW) when fruit were stored at 20 °C. Beta-carotene peaked at 40% of total carotenoids when fruit were stored at 4 °C. The levels of alpha-carotene were barely detectable and did not show any variation in fruit stored at either 4 °C or 20 °C. Lutein levels increased from 1.89±0.5 to 2.56±0.21 μg g⁻¹ FW in fruit stored at 20 °C, while at 4 °C lutein increased to 3.42±0.61 μg g⁻¹ FW, indicating that temperature does not significantly influence the accumulation of this compound.

**Gene expression in A. macrosperma stored at different temperatures**

Gene expression of *PSY*, *PDS*, *ZDS*, *CRTISO*, *LCY-β*, *LCY-ε*, *CRH-β*, and *CRH-ε* was examined in *A. macrosperma* fruit at different temperatures. In general, the relative expression of these genes was reduced in fruit stored at 4 °C compared with 20 °C (Fig. 10). However, there were only slight changes in expression of *PDS*, *CRTISO*, and *CRH-ε*, when the fruit were stored or transferred between the different temperature treatments (Fig. 10). *PSY*, *ZDS*, and *CRH-β* expression increased steadily in the fruit stored...
at 20 °C, but in fruit at 4 °C there was a reduction in gene expression that appeared to be reversed when fruit were transferred to 20 °C. The effect of these treatments on the relative expression of LCY-β was more pronounced than on PSY, ZDS, and CRH-β. There was a 20-fold increase in the expression of LCY-β when fruit were stored at 20 °C over the course of the experiment. At 4 °C, an initial increase in relative gene expression was followed by a significant reduction in gene expression. When fruit were moved from 4 °C to 20 °C, the LCY-β expression level increased by ~13 fold (Fig. 10).

**Actinidia LCY-β encodes a functional biosynthetic enzyme**

While sequence similarity can give an indication of the enzymatic function of the encoded protein, this cannot be assumed. To confirm the enzyme activity of Actinidia LCY-β in carotenoid biosynthesis, lycopene-producing bacteria (Cunningham and Gantt, 1998) were transformed with a vector carrying the AdLCY-β1 cDNA and grown at 28 °C for 2 d. The colour of the transformed bacteria culture changed from red to orange, suggesting the conversion of lycopene to beta-carotene (Fig. 11A).

The substrates for LCY-β enzyme are lycopene, which is converted to beta-carotene, and delta-carotene which is converted to alpha-carotene. To confirm the activity of the purified recombinant LCY-β, the full-length open reading frame (813 bp) of kiwifruit LCY-β, was cloned in a translational fusion into pET30b expression vector with a c-terminal HIS6 tag (Novagen). The purified recombinant protein from Escherichia coli was analysed by SDS-PAGE (data not shown) and showed the presence of a protein of molecular mass 57 kDa, which was consistent with the predicted mass based on its sequence. The recombinant enzyme was used in *in vitro* assays with lycopene and delta-carotene as substrates (Schnurr *et al.*, 1996). The presence of beta-carotene and alpha-carotene, when lycopene and delta-carotene were used as substrates respectively (Fig. 11B), was confirmed by HPLC. These results demonstrate that this *Actinidia* LCY-β is able to catalyse the biosynthetic reaction predicted by its sequence similarity.

**Discussion**

**The dominant carotenoid in kiwifruit is beta-carotene**

Beta-carotene was detected in all the kiwifruit genotypes studied and was the dominant carotenoid accumulating in fruit. In contrast, alpha-carotene was at very low levels. Among the xanthophylls accumulating in kiwifruit, lutein was present at relatively high levels, while the beta-xanthophylls such as zeaxanthin and antheraxanthin were present at very low levels. The high levels of beta-carotene and lutein are similar to those described in other fruit crops.

![Fig. 8. Photograph of Actinidia macrosperma fruit colour development. (A) At harvest, (B) following 38 d storage at 4 °C, (C) following storage of 27 d at 20 °C, and (D) 20 d at 4 °C then 18 d at 20 °C.](https://academic.oup.com/jxb/article-abstract/60/13/3765/529882)
such as capsicum, mango, and papaya (Gouado et al., 2007; Ha et al., 2007; Veda et al., 2007).

In all the kiwifruit selections studied, the linear carotenoids such as phytoene, zeta-carotene, and lycopene were not detected. These linear carotenoids are, however, found to accumulate to higher concentrations in other fruits (Yano et al., 2005; Zhou et al., 2007). It is not clear what specifies the types of compounds that accumulate in different species. However, low concentrations of linear carotenoids could be due to efficiency of enzymatic conversion of these molecules or an indication of an upstream limiting factor. In kiwifruit, the cDNA of phytoene synthase was not found in an EST database of more than 100,000 sequences, so a degenerate PCR approach was required to clone this gene (Crowhurst et al., 2008). This enzyme, committed to the condensation of geranyl geranyl pyrophosphate to form phytoene is thought to be a rate-limiting factor in carotenoid biosynthesis (Giuliano et al., 1993; Fraser et al., 2002). However, downstream carotenoid compounds were found to accumulate to high levels in kiwifruit, suggesting that the first enzyme step is active in this species.

The accumulation of downstream compounds such as beta-carotene and lutein in kiwifruit, combined with the undetectable levels of phytoene, zeta-carotene, and lycopene, suggest efficient enzyme activities at those intermediate steps. In tomato, the early enzyme steps have a significant influence over the flux. The activities of the desaturation enzymes in the pathway leading to lycopene seem to increase while the cyclization steps, downstream of lycopene, are reduced resulting in lycopene accumulation (Fraser et al., 1994, 2002). Similarly, when transgenes of PSY and CRTI (Erwinia uredovora) were introduced into rice, there was accumulation of beta-carotene instead of the expected lycopene, indicating the presence of an efficient endogenous cyclase activity in rice (Al-Babili et al., 2001; Paine et al., 2005; Schaub et al., 2005).

Although the mechanisms that influence the level and distribution of carotenoid or the relative distribution of carotenoid compounds are not well known, it can be hypothesized that the relative activities of LCY-β and LCY-e might serve to apportion the flux into the two branches of the pathway (Cunningham et al., 1996; Pecker

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**Fig. 9.** The accumulation of carotenoids in *Actinidia macrosperma* ripe and stored fruit. Concentrations of β-carotene (A–C) and lutein (D–F) levels in fruit: error bars are standard errors of the mean from three independent measurements. Fruit were stored at 20 °C (A, D), 4 °C (B, E) or at 4 °C later transferred to 20 °C (C, F). Whole fruits (skin+pericarp) were sampled at mature green stage (T₀) and thereafter at various intervals (T₁, T₂, T₃) for the three comparable timepoints during the ripening stages. The T₁, T₂, T₃ time points at 20 °C were 10, 15, and 27 d storage; at 4 °C were 20, 29, and 38 d. For the transfer of fruit between 4 °C and 20 °C, 20 d at 4 °C (T₁) followed by 9 d (T₂), 18 d (T₃) at 20 °C, respectively.
et al., 1996). In this study, it was found that the concentrations of beta-carotene in kiwifruit correlated with the gene expression levels of LCY-b. This suggests that LCY-b plays a significant role in apportioning metabolites to the β, β ring branch of the carotenoid pathway.

**Carotenoid accumulation varies with genotype and developmental stage**

Substantial genotypic differences in carotenoid accumulation were observed between the kiwifruit genotypes in this study. Total carotenoids, measured by HPLC, showed that the *A. chinensis* genotypes analysed had low concentrations of carotenoids. By contrast, *A. macrosperma* and the three individuals of the *A. macrosperma*A. *melanandra* population accumulated higher concentrations of carotenoids in the fruit (Montefiori et al., 2005). Beta-carotene accumulated to ~46 μg g⁻¹ FW in *A. macrosperma*, which is more than 90-fold greater than in the *A. chinensis* individuals used in this study. To help understand the differences among these genotypes, the expression levels of kiwifruit cDNAs with similarity to the known carotenoid biosynthetic genes were examined. Of these, the lycopene beta-cyclase gene (LCY-b) appeared to show alterations in expression patterns that correlated with the diverse range of distribution of beta-carotene within the various kiwifruit genotypes.

Differences in carotenoid content, attributed to the LCY-b genes in *Actinidia* species may be due either to different enzymatic activities or to altered levels of LCY-b expression. The cDNA sequences of LCY-b genes from *A. macrosperma*, *A. melanandra*, *A. delicosa*, and *A. chinensis* were further examined and within the open reading frames there were slight differences. All had similar sequence lengths, with the exception of *A. chinensis* which had an extra 21 base pairs.

![Fig. 10. Expression of PSY, PDS, ZDS, CRTISO, LCY-β, LCY-ε, CRH-β, and CRH-ε in Actinidia macrosperma fruit ripening at different temperatures. RNA was isolated from whole fruit (skin+pericarp) at T₀, T₁, T₂, and T₃ timepoints. The T₁, T₂, T₃ time points at 20 °C were 10, 15, and 27 d storage; at 4 °C were 20, 29, and 38 d. For the transfer of fruit between 4 °C and 20 °C, 20 d at 4 °C (T₁) followed by 9 d (T₂), 18 d (T₃) at 20 °C, respectively.](https://academic.oup.com/jxb/article-abstract/60/13/3765/529882)
The similarity among predicted amino acid sequences were 94% or more. However, a more detailed kinetic analysis of the various LCY-β enzymes is required to determine if these differences are responsible for the accumulation pattern of beta-carotene in the kiwifruit genotypes.

The concentration of beta-carotene generally increased with developmental stage. Carotenoids accumulated to a greater concentration during fruit ripening. This is consistent with the pattern observed in other plants such as mango, pepper, and papaya in which carotenoid accumulation is developmentally regulated (Fabi et al., 2007; Ha et al., 2007). Kiwifruit stored at room temperature ripened quickly and accumulated carotenoids to high concentrations while cold storage inhibited carotenoid accumulation. Apparently, the conditions that inhibit the fruit-ripening process also inhibit carotenoid accumulation, as observed in other fruit types (Neta-Sharir et al., 2005; Gil et al., 2006; Perkins-Veazie and Collins, 2006). However, little is known about the molecular mechanism regulating these events. In this study, it was found that expression of key biosynthetic genes were temperature sensitive and account for these carotenoid accumulation patterns. This has significance for the post-harvest storage of fruit and its effect on market quality.

**Carotenoid accumulation in kiwifruit is controlled by gene expression**

The expression of the carotenoid biosynthetic genes explains the different carotenoid accumulation patterns seen in the kiwifruit genotypes. Expression of ZDS and LCY-β transcripts increased in kiwifruit genotypes and at developmental stages where carotenoid concentrations were high. LCY-β gene expression in the MaMe genotypes increased from the early fruit stage through to ripening and clearly differed between the high and low carotenoid individuals. In contrast, PSY, PDS, CRTISO, and CRH-β expression levels were not responsive and had similar profiles among all three genotypes, suggesting that they are not key determinants of carotenoid accumulation in kiwifruit. Similarly, in Actinidia macroasperma fruit, PDS, CRTISO, LCY-e, and CRH-e appeared to be non-responsive or were down-regulated by the temperature treatment that resulted in increased fruit carotenoids, while PSY, ZDS, LCY-β, and CRH-β expression increased in response to the conditions that consequently increased carotenoid accumulation. The relationship between carotenoid accumulation and gene expression has been investigated in various crop species. In citrus (orange), beta-carotene accumulation was found to be directly related to expression of PDS rather than LCY-β (Fanciullino et al., 2008). In addition, in pepper, high concentrations of carotenoids were associated with high expression of PSY and PDS, although only a limited number of carotenoid biosynthetic genes were tested (Ha et al., 2007). In potato, high transcript levels of PSY were observed in the high carotenoid accession compared with the low carotenoid accession, while the expression level of LCY-β was similar in all the genotypes (Morris et al., 2004). These results combine to suggest that carotenoid...
biosynthesis may be controlled differently in different species of plants. Although, changes in transcript abundance can be due either to increased transcription or altered mRNA stability, our data suggest that, in \textit{Actinidia} species, carotenoid accumulation may depend largely on transcriptional regulation of different alleles of the carotenoid biosynthetic genes.

In conclusion, our study of carotenoids in kiwifruit found significant differences in carotenoid accumulation patterns among kiwifruit genotypes. The major carotenoid was found to be beta-carotene and its accumulation was controlled by the increased expression of the ZDS and \textit{LCY-\beta}\textsubscript{+} genes and the down-regulation of \textit{LCY-e} and \textit{CRH-\beta} genes, suggesting these are important regulatory steps in the kiwifruit carotenoid biosynthetic pathway. The differences in gene expression observed among the genotypes may be due to differential regulation of these genes, which requires further investigation to help us understand fruit carotenoid accumulation in kiwifruit.

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