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RESEARCH PAPER

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

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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*-ϵ) 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 colourless phytoene is subsequently desaturated to give zeta-carotene and lycopene (Norris *et al.*, 1995). In bacteria, the desaturation of phytoene is by one enzyme, carotene desaturase (CrtI) but in plants, two enzymes, phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) are required (Bartley *et al.*, 1999). The carotenoid pathway branches at the cyclization of lycopene, which is acted upon

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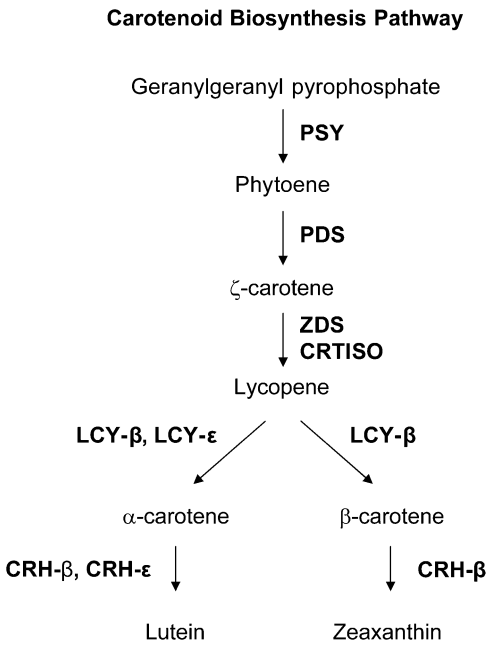

Fig. 1. Carotenoid biosynthetic pathway in plants. Enzymatic conversions are shown by arrows with the enzymes responsible in bold. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zeta-carotene desaturase; CRTISO, carotene isomerase; LCY-β, lycopene beta-cyclase; LCY-ε, lycopene epsilon-cyclase;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

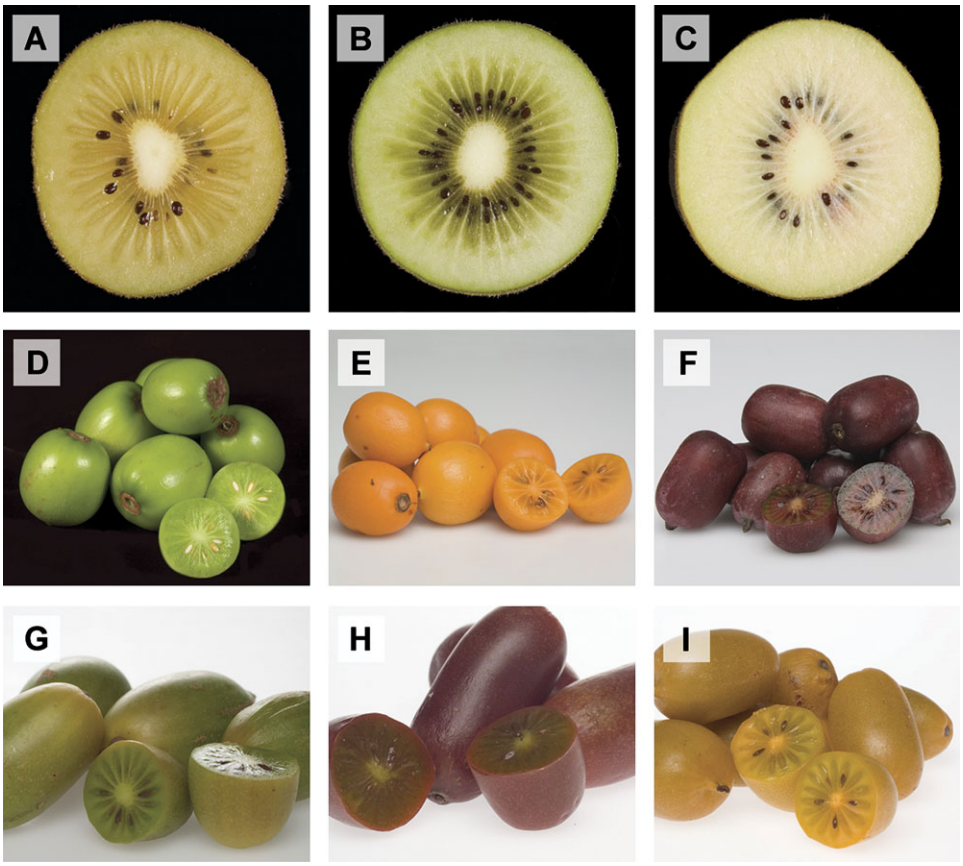

Fig. 2. Fruit of the *Actinidia* genotypes used in this study. (A) *A. chinensis* MP161, (B) *A. chinensis* MP165, (C) *A. chinensis* MP214, (D) *A. macrosperma* mature, (E) *A. macrosperma* ripe, (F), *A. melanandra*. Progeny from a cross between *A. macrosperma*×*A. melanandra*: (G) MaMe1, (H) MaMe2, and (I) MaMe3.

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 $L{\times}a{\times}b$ 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 CATTTGGGGGCTTTGGGTTGTGT and F2 TGCAGTTCGGGACCTTAAGAAACTC in combination with GeneRacer (Invitrogen) primers 3′ P

GCTGTCAACGATACGCTACGTAACG and 3′ NP CGCTACGTAACGGCATGACAGTG, respectively.

Primers for *Actinidia* carotenoid biosynthetic genes were designed across predicted intron positions of each gene with Tm of ~60 °C. Primer pairs used for amplification were as follows: Actin (TGCATGAGCGATCAAGTTTCAAG, TGTCCCATGTCTGGTTGATGACT), PSY (CGAGATTGAAGCCAACGACTAC, GTTCTCGAAGGGGCAACAATAG) PDS (AGCAGAAGCCCCCTTCTC AGTG, TCCTCTGCAGGTGCAAAAACCA), ZDS (TGCATTGTTTGCCACCAAAACAG, TGCATCCCCACCTGAGATGAAA), CRTISO (GGACACCAAAGACACACAGGAG, GTTGTGTTGAATGGCATCCCTA), LCY-β (GTCGTTCCCGATTCGACGTGAT, TGAAA GTGGCGAGGGATCAACA), LCY-ε (TCGGGTCTA CTCTCTCCTCAGC, GGTCGGAAAGTAGATGCCTGAT), CRH-β (AGAATCGCATGGCGAAGAGGAG, GGACATGACTGCAGCGACAAGG), CRH-ε (AGGTCCACCACTAAATGGGATG, AGGTCTGGGAGAGAGCAGAAGA).

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 into 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 *Nde*I and *Kpn*I 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 OD_{600} 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-Bind 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 His 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 A280 nm) were identified.

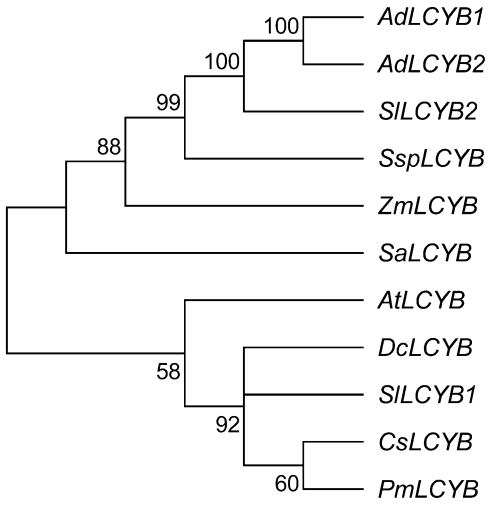

Fig. 3. A phylogenetic tree of lycopene beta-cyclase using amino acid sequences of the species shown. The two non-redundant kiwifruit sequences were most similar to the tomato chromoplast-specific sequence. The GenBank accession numbers of the proteins, or translated products, used in the analysis are as follows: *PmLCYB, Prunus mume* (plum) [BAF49055]; *CsLCYB, Citrus sinensis* (orange) [ABB72443]; *DcLCYB, Daucus carota* (carrot) [ABB52071]; *SlLCYB1, Solanum lycopersicum* 1 (tomato) [Q43503]; *SlLCYB2, Solanum lycopersicum* 2 (tomato; chromoplast specific) [AAG21133]; *AtLCYB, Arabidopsis thaliana* (thale cress) [AT3G10230], *ZmLCYB, Zea mays* (maize) [AAO18661]; *SaLCYB, Sandersonia aurantiaca* (Chinese lily/Christmas-bells) [AAL92175]; *AdLCYB1, Actinidia deliciosa* 1 (kiwifruit) [FJ427508]; *AdLCYB2, Actinidia deliciosa* 2 (kiwifruit) [FJ427509]; *SspLCYB, Synechococcus* sp (cyanobacteria) [YP001226999].

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 electrotransferred to a nitrocellulose filter for Western analysis. His-tagged proteins were bound with mouse anti HIS_6 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 $FeSO_3$

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 N_2.

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 Na_2CO_3 and 500 mg of anhydrous Na_2SO_4. 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 N_2.

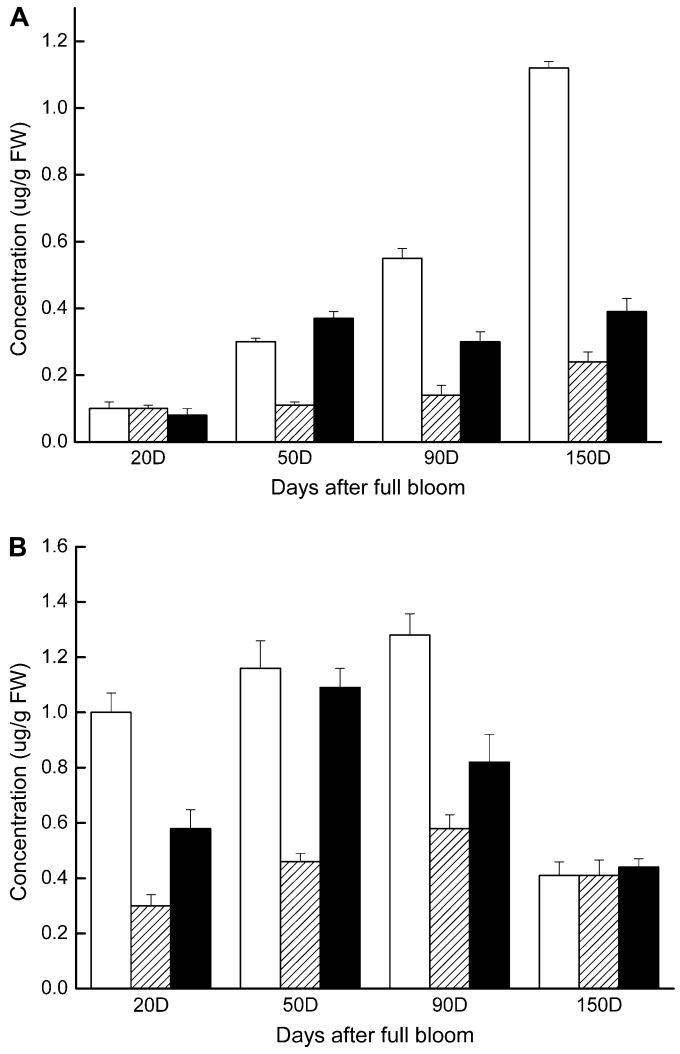

Fig. 4. Accumulation of beta-carotene (A) and lutein (B) in *Actinidia chinensis* genotypes; MP161 (white bars), MP165 (hatched bars), and MP214 (black bars). Fruits were picked at developmental stages 20, 50, 90, and 150 d after full bloom and the pericarp analysed for their carotenoid concentration using HPLC. Error bars are standard errors of the mean of three technical samples.

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 Chromeleon 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

Table 1. Total carotenoid and chlorophyll levels in *Actinidia chinensis* (µg g^{-1} fresh weight) by HPLC

Carotenoid and chlorophyll concentrations measured in fruit of three *Actinidia chinensis* individuals (MP161, MP165, and MP214) at 50, 90, and 150 d after full bloom (DAFB). Data represent mean from four replicates.

	Fruit genotypes								
	Mp161			Mp165			Mp214		
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

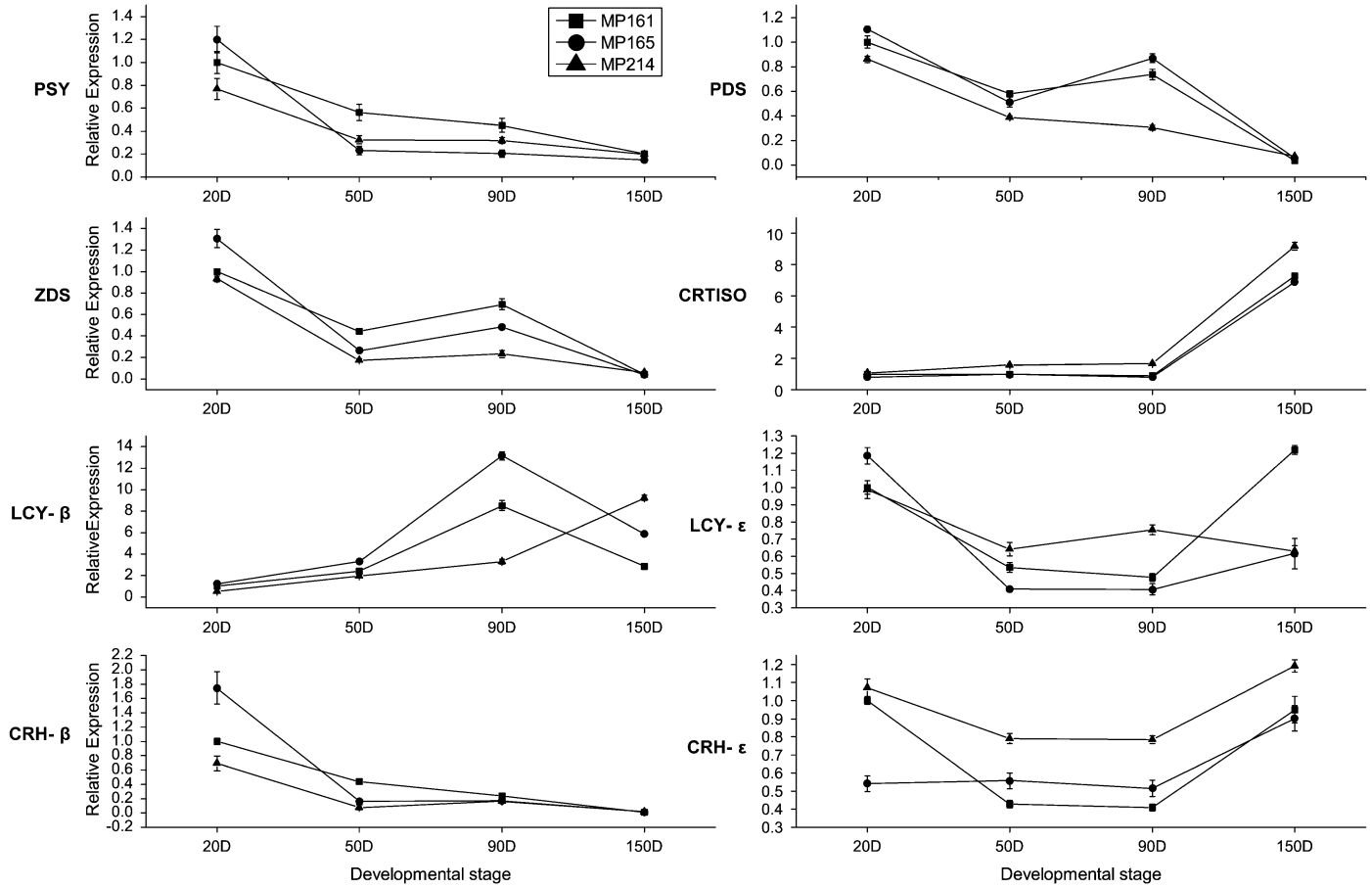

Fig. 5. Expression of carotenoid biosynthetic genes in three *Actinidia chinensis* genotypes MP161, MP165, and MP214. The genes were phytoene synthase (*PSY*), phytoene desaturase (*PDS*), zeta-carotene desaturase (*ZDS*), carotenoid isomerase (*CRTISO*), lycopene beta-cyclase (*LCY-β*), lycopene epsilon-cyclase (*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.

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 deliciosa* 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-β1* and *AdLCY-β2* 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-β1* (FJ427508) showed 56% identity to *Arabidopsis* (AT3G10230), 53% to carrot LCY-β (ABB52071) and was phylogenetically more closely related to the tomato chromoplast-specific LCY-β (AAG21133), than the other LCY-βs 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

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-β*, *LCY-ε* *CRH-β*, and *CRH-ε* 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 carotenes 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 fruit (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-β* 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-β* 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-β*, 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-β, LCY-ε*, and *CRH-ε* expressions, which differed significantly among the three genotypes especially at 150 DAFB.

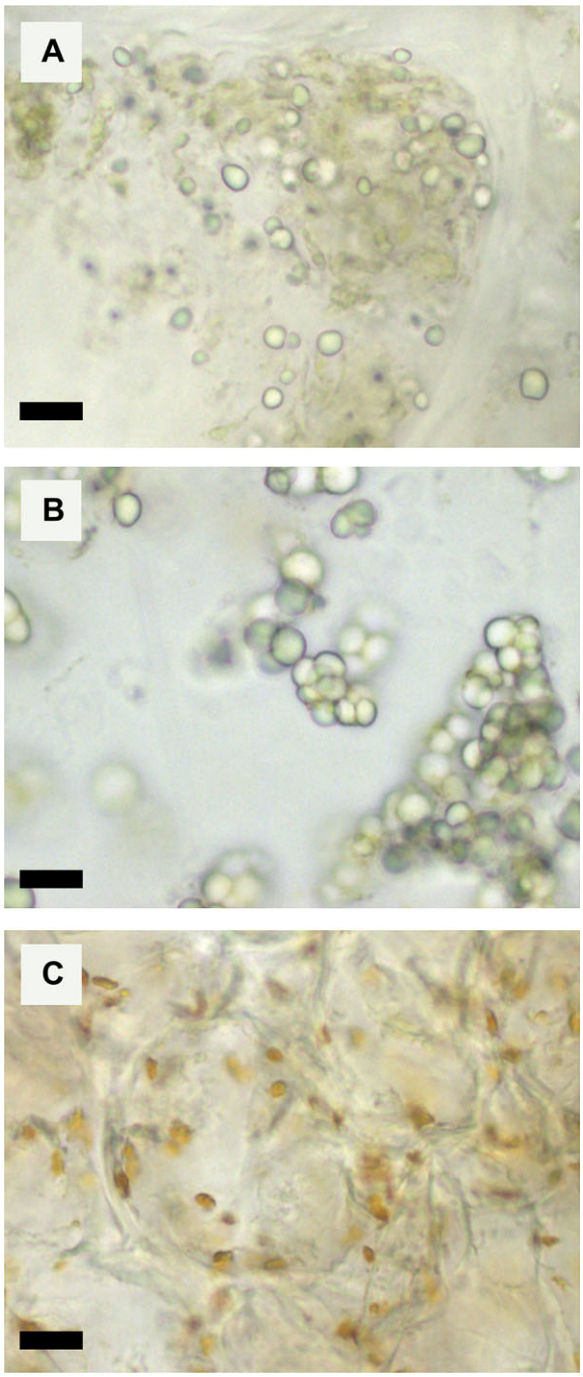

Fig. 6. Light micrographs of ripe fruit pericarp from individuals of an *Actinidia macrosperma*×*A. melanandra* cross. (A) MaMe1, (B) MaMe2, and (C) MaMe3 showing orange-coloured chromoplasts. Scale bar=10 μm.

Carotenoid accumulation in a cross between A. macrosperma *and* A. melanandra

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

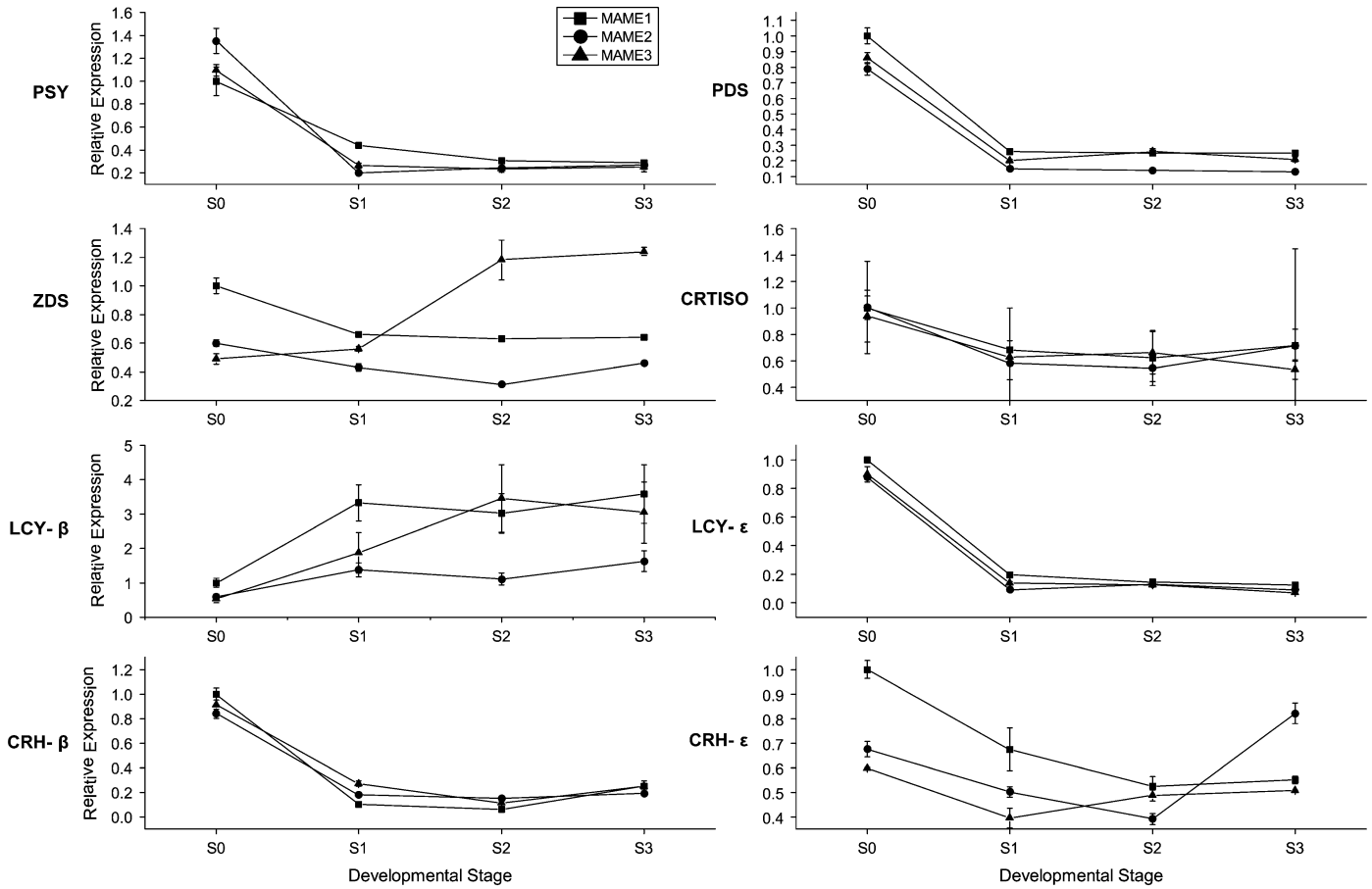

Fig. 7. Expression of carotenoid biosynthetic genes in individuals of an *Actinidia macrosperma*×*A. melanandra* cross: MaMe1, MaMe2, and MaMe3. Fruit stages were 20 DAFB (S0), matured green (S1), 50% ripe stage (S2), and fully ripe (S3). Gene expression was analysed at the same stage as the carotenoid measurements in Table 2.

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 T_0, to 46.99±15.12 μg g^{-1} FW at T_3 (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 T_3, 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 T_0, 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

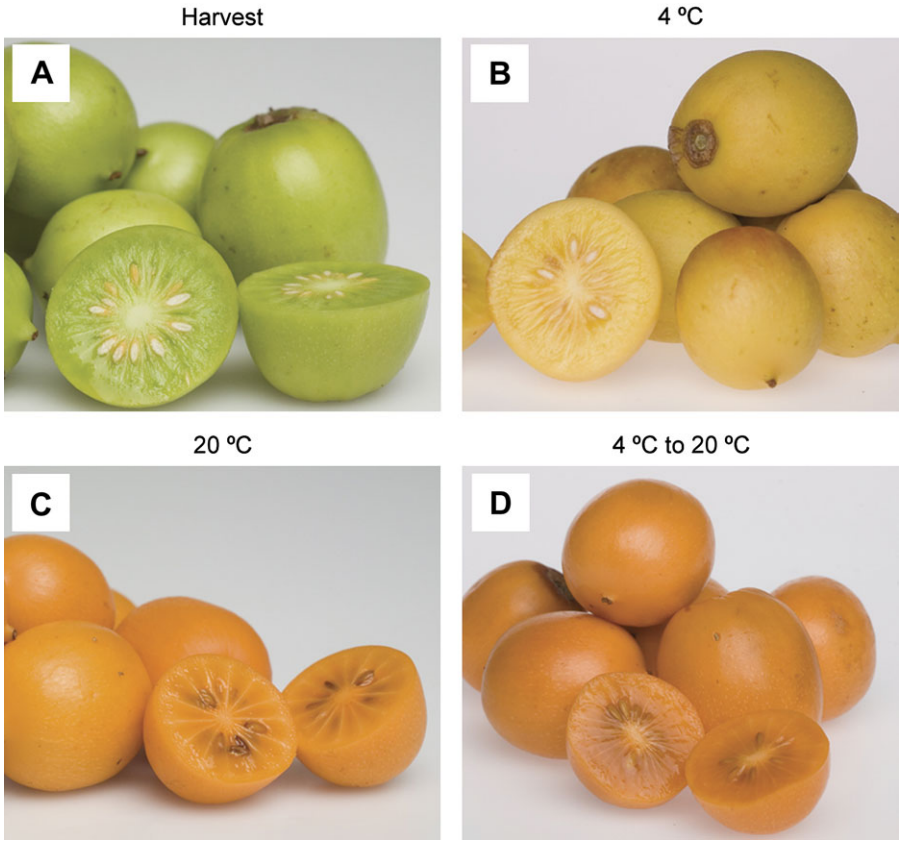

Fig. 8. Photograph of *Actinidia macrosperma* fruit colour development. (A) At harvest, (B) following 38 d storage at 4 °C, (C) following storage of 27 d at 20 °C, and (D) 20 d at 4 °C then 18 d at 20 °C.

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 HIS_6 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

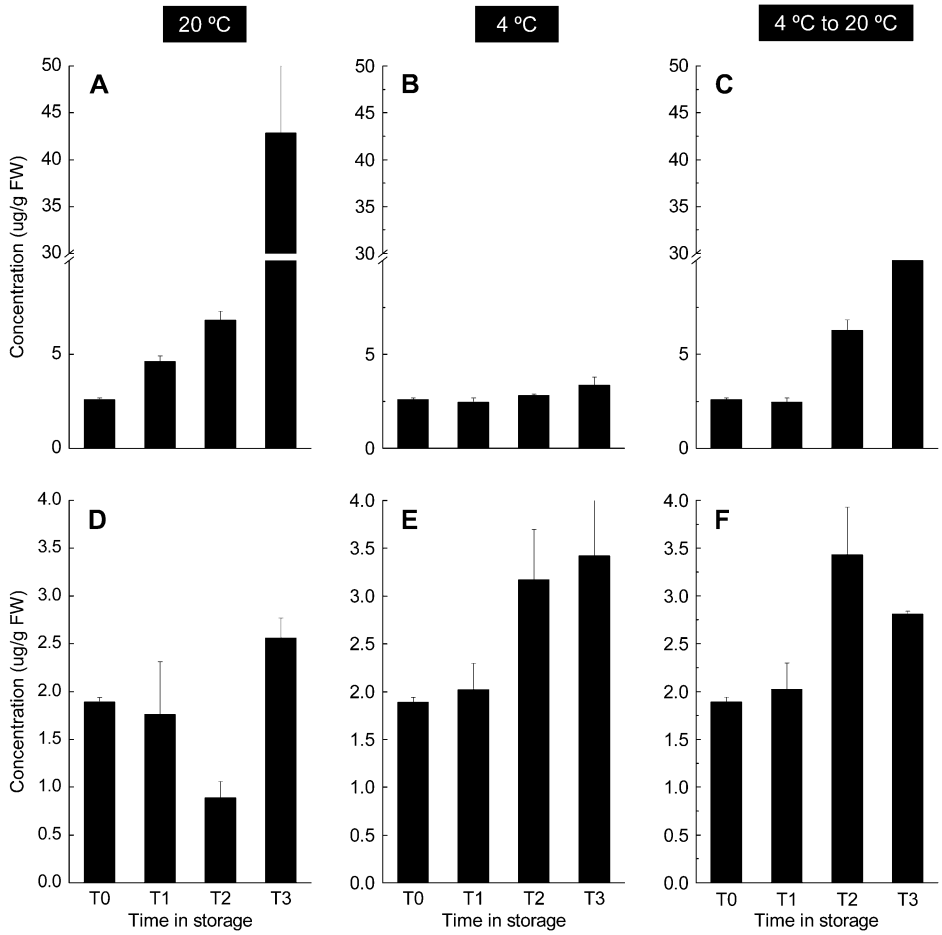

Fig. 9. The accumulation of carotenoids in *Actinidia macrosperma* ripe and stored fruit. Concentrations of β-carotene (A–C) and lutein (D–F) levels in fruit: error bars are standard errors of the mean from three independent measurements. Fruit were stored at 20 °C (A, D), 4 °C (B, E) or at 4 °C later transferred to 20 °C (C, F). Whole fruits (skin+pericarp) were sampled at mature green stage (T_0) and thereafter at various intervals (T_1, T_2, T_3) for the three comparable timepoints during the ripening stages. The T_1, T_2, T_3 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 (T_1) followed by 9 d (T_2), 18 d (T_3) at 20 °C, respectively.

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 carotenes such as phytoene, zeta-carotene, and lycopene were not detected. These linear carotenes 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-ε might serve to apportion the flux into the two branches of the pathway (Cunningham *et al.*, 1996; Pecker

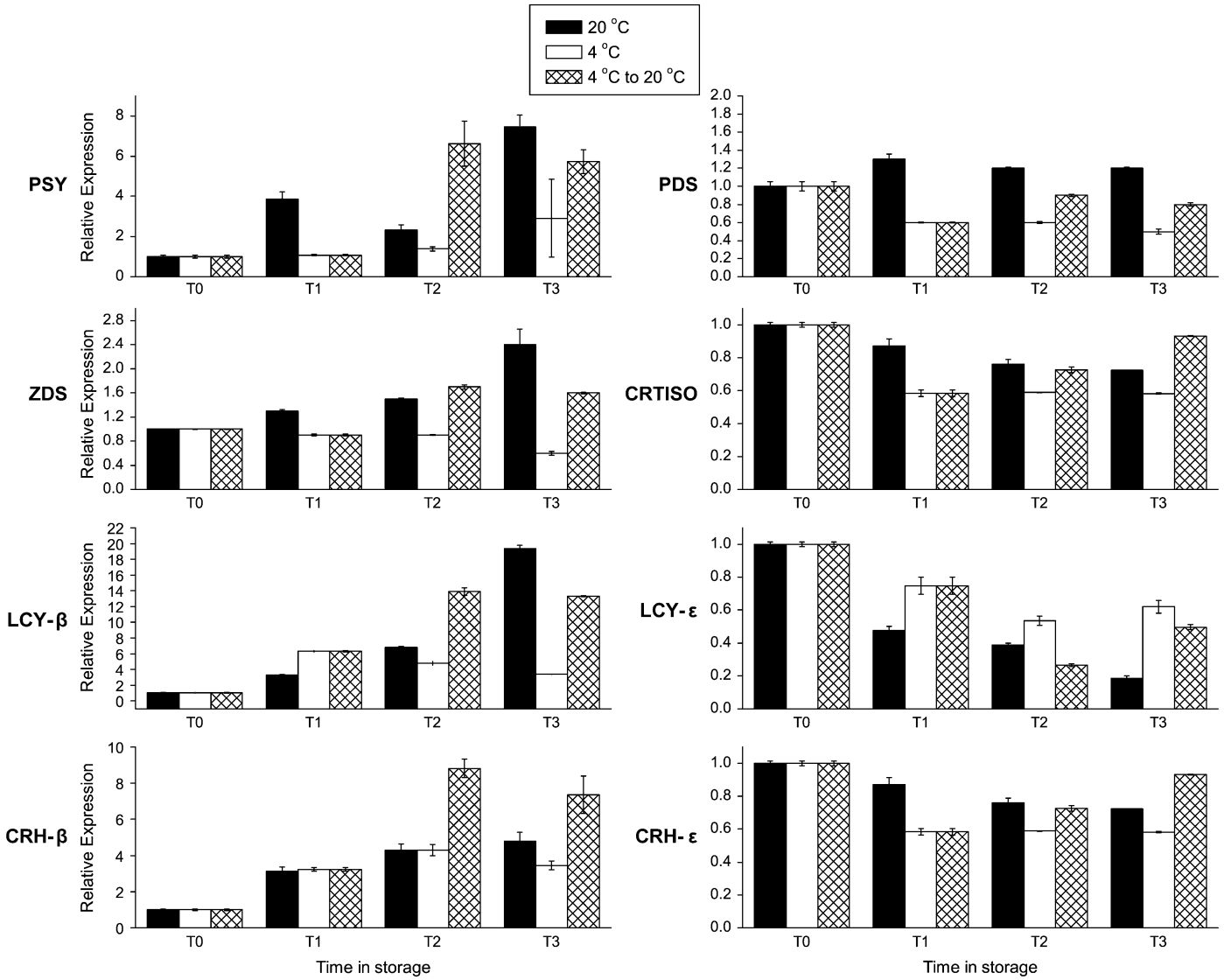

Fig. 10. Expression of *PSY*, *PDS*, *ZDS*, *CRTISO*, *LCY-β*, *LCY-ε*, *CRH-β*, and *CRH-ε* in *Actinidia macrosperma* fruit ripening at different temperatures. RNA was isolated from whole fruit (skin+pericarp) at T_0, T_1, T_2, and T_3 timepoints. The T_1, T_2, T_3 time points at 20 °C were 10, 15, 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 (T_1) followed by 9 d (T_2), 18 d (T_3) 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-β*. This suggests that LCY-β 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-β*) 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-β* genes in *Actinidia* species may be due either to different enzymatic activities or to altered levels of *LCY-β* expression. The cDNA sequences of *LCY-β* genes from *A. macrosperma*, *A. melanandra*, *A. deliciosa*, 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.

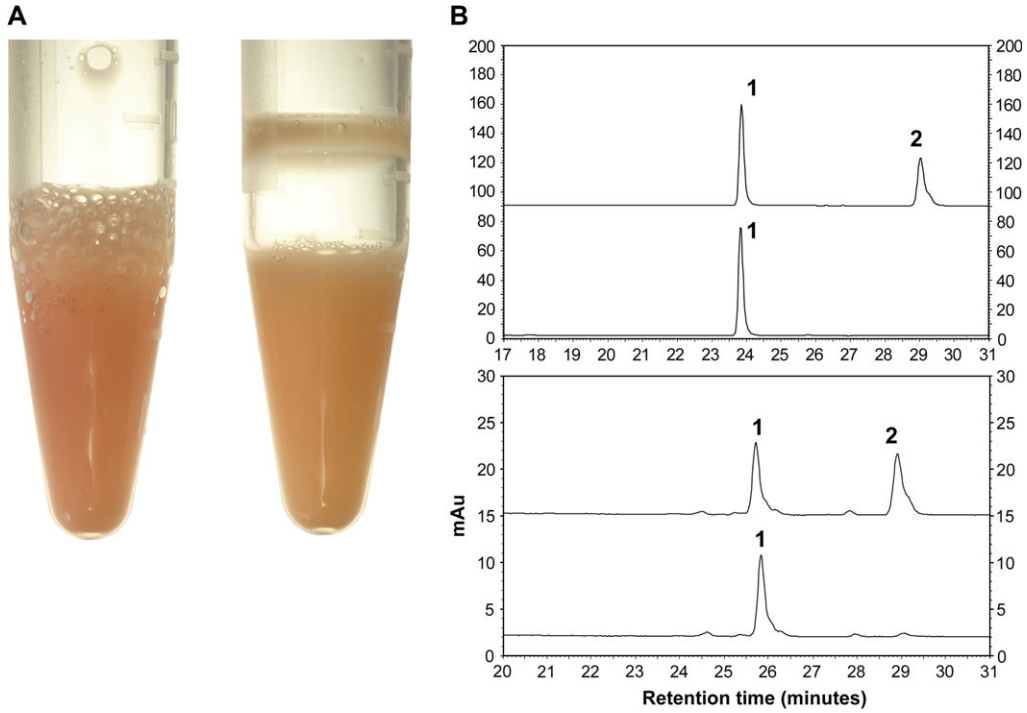

Fig. 11. *In vitro* activity of recombinant kiwifruit lycopene β-cyclase (LCY-β). (A) Lycopene-producing bacteria transformed with *AdLCY-β1* cDNA showed colour change from red (left) to orange (right) after growing at 28 °C for 2 d, suggesting the conversion of lycopene to beta-carotene. (B) Conversion of lycopene and delta-carotene by kiwifruit AdLCY-β1 in the enzyme assay. Top chart, chromatogram showing lycopene substrate (peak 1) and with beta-carotene (peak 2) after assay; lower chart, delta-carotene (peak 1) and with alpha-carotene (peak 2) after assay.

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 *A. macrosperma* fruit, *PDS*, *CRTISO*, *LCY-ε*, and *CRH-ε* 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 *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 *LCY-β* genes and the down-regulation of *LCY-ε,* and *CRH-β* 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.

Acknowledgements

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References

Al-Babili S, Hoa TT, Schaub P. 2006. Exploring the potential of the bacterial carotene desaturase CrtI to increase the beta-carotene content in Golden Rice. *Journal of Experimental Botany* **57,** 1007–1014.

Al-Babili S, Ye X, Lucca P, Potrykus I, Beyer P. 2001. Biosynthesis of beta-carotene (provitamin A) in rice endosperm achieved by genetic engineering. *Novartis Foundation Symposium* **236,** 219–228; discussion 228–232.

Aluru M, Xu Y, Guo R, Wang Z, Li S, White W, Wang K, Rodermel S. 2008. Generation of transgenic maize with enhanced provitamin A content. *Journal of Experimental Botany* **59,** 3551–3562.

Bartley GE, Scolnik PA, Beyer P. 1999. Two *Arabidopsis thaliana* carotene desaturases, phytoene desaturase and zeta-carotene desaturase, expressed in *Escherichia coli*, catalyse a poly-*cis* pathway to yield pro-lycopene. *European Journal of Biochemistry* **259,** 396–403.

Beyer P, Al-Babili S, Ye X, Lucca P, Schaub P, Welsch R, Potrykus I. 2002. Golden Rice: introducing the beta-carotene biosynthesis pathway into rice endosperm by genetic engineering to defeat vitamin A deficiency. *Journal of Nutrition* **132,** 506S–510S.

Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **116,** 113–116.

Cheng CH, Seal AG, Boldingh HL, Marsh KB, MacRae EA, Murphy SJ, Ferguson AR. 2004. Inheritance of taste characters and fruit size and number in a diploid *Actinidia chinensis* (kiwifruit) population. *Euphytica* **138,** 185–195.

Crowhurst RN, Gleave AP, MacRae EA, *et al*. 2008. Analysis of expressed sequence tags from *Actinidia*: applications of a cross species EST database for gene discovery in the areas of flavor, health, color and ripening. *BMC Genomics* **9,** 351.

Cunningham FX, Gantt E. 1998. Genes and enzymes of carotenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **49,** 557–583.

Cunningham FX, Pogson B, Sun Z, McDonald KA, DellaPenna D, Gantt E. 1996. Functional analysis of the beta and epsilon lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *The Plant Cell* **8,** 1613–1626.

Cunningham FX, Sun Z, Chamovitz D, Hirschberg J, Gantt E. 1994. Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp. strain PCC7942. *The Plant Cell* **6,** 1107–1121.

Davison PA, Hunter CN, Horton P. 2002. Overexpression of beta-carotene hydroxylase enhances stress tolerance in Arabidopsis. *Nature* **418,** 203–206.

DellaPenna D, Pogson BJ. 2006. Vitamin synthesis in plants: tocopherols and carotenoids. *Annual Review of Plant Biology* **57,** 711–738.

Dong H, Deng Y, Mu J, Lu Q, Wang Y, Xu Y, Chu C, Chong K, Lu C, Zuo J. 2007. The Arabidopsis Spontaneous Cell Death1 gene, encoding a zeta-carotene desaturase essential for carotenoid biosynthesis, is involved in chloroplast development, photoprotection and retrograde signalling. *Cell Research* **17,** 458–470.

Fabi JP, Cordenunsi BR, de Mattos Barreto GP, Mercadante AZ, Lajolo FM, Oliveira do Nascimento JR. 2007. Papaya fruit ripening: response to ethylene and 1-methylcyclopropene (1-MCP). *Journal of Agricultural and Food Chemistry* **55,** 6118–6123.

Fanciullino AL, Cercos M, Dhique M, Froelicher Y, Talon M, Ollitrault P, Morillon R. 2008. Changes in carotenoid content and biosynthetic gene expression in juice sacs of four orange varieties (*Citrus sinensis*) differing in flesh fruit color. *Journal of Agricultural and Food Chemistry* **56,** 3628–3638.

Fraser LG, Harvey CF, Crowhurst RN, De Silva HN. 2004. EST-derived microsatellites from *Actinidia* species and their potential for mapping. *Theoretical and Applied Genetics* **108,** 1010–1016.

Fraser PD, Romer S, Shipton CA, Mills PB, Kiano JW, Misawa N, Drake RG, Schuch W, Bramley PM. 2002. Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. *Proceedings of the National Academy of Sciences, USA* **99,** 1092–1097.

Fraser PD, Truesdale MR, Bird CR, Schuch W, Bramley PM. 1994. Carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). *Plant Physiology* **105,** 405–413.

Gil MI, Aguayo E, Kader AA. 2006. Quality changes and nutrient retention in fresh-cut versus whole fruits during storage. *Journal of Agricultural and Food Chemistry* **54,** 4284–4296.

Giuliano G, Bartley GE, Scolnik PA. 1993. Regulation of carotenoid biosynthesis during tomato development. *The Plant Cell* **5,** 379–387.

Gouado I, Schweigert FJ, Ejoh RA, Tchouanguep MF, Camp JV. 2007. Systemic levels of carotenoids from mangoes and papaya consumed in three forms (juice, fresh and dry slice). *European Journal of Clinical Nutrition* **61,** 1180–1188.

Ha SH, Kim JB, Park JS, Lee SW, Cho KJ. 2007. A comparison of the carotenoid accumulation in *Capsicum* varieties that show different ripening colours: deletion of the capsanthin-capsorubin synthase gene is not a prerequisite for the formation of a yellow pepper. *Journal of Experimental Botany* **58,** 3135–3144.

Harjes CE, Rocheford TR, Bai L, *et al*. 2008. Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. *Science* **319,** 330–333.

Hirschberg J. 2001. Carotenoid biosynthesis in flowering plants. *Current Opinion in Plant Biology* **4,** 210–218.

Humphries JM, Graham RD, Mares DJ. 2004. Application of reflectance colour measurement to the estimation of carotene and lutein content in wheat and triticale. *Journal of Cereal Science* **40,** 151–159.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5,** 150–163.

McGhie TK, Ainge GD. 2002. Color in fruit of the genus *Actinidia*: carotenoid and chlorophyll compositions. *Journal of Agricultural and Food Chemistry* **50,** 117–121.

Montefiori M, McGhie TK, Costa G, Ferguson AR. 2005. Pigments in the fruit of red-fleshed kiwifruit (*Actinidia chinensis* and *Actinidia deliciosa*). *Journal of Agricultural and Food Chemistry* **53,** 9526–9530.

Morris WL, Ducreux L, Griffiths DW, Stewart D, Davies HV, Taylor MA. 2004. Carotenogenesis during tuber development and storage in potato. *Journal of Experimental Botany* **55,** 975–982.

Neta-Sharir I, Isaacson T, Lurie S, Weiss D. 2005. Dual role for tomato heat shock protein 21: protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. *The Plant Cell* **17,** 1829–1838.

Norris SR, Barrette TR, DellaPenna D. 1995. Genetic dissection of carotenoid synthesis in arabidopsis defines plastoquinone as an essential component of phytoene desaturation. *The Plant Cell* **7,** 2139–2149.

Paine JA, Shipton CA, Chaggar S, *et al*. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nature Biotechnology* **23,** 482–487.

Pecker I, Gabbay R, Cunningham FX Jr, Hirschberg J. 1996. Cloning and characterization of the cDNA for lycopene beta-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Molecular Biology* **30,** 807–819.

Perkins-Veazie P, Collins JK. 2006. Carotenoid changes of intact watermelons after storage. *Journal of Agricultural and Food Chemistry* **54,** 5868–5874.

Pogson B, McDonald KA, Truong M, Britton G, DellaPenna D. 1996. Arabidopsis carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. *The Plant Cell* **8,** 1627–1639.

Schaub P, Al-Babili S, Drake R, Beyer P. 2005. Why is golden rice golden (yellow) instead of red? *Plant Physiology* **138,** 441–450.

Schnurr G, Misawa N, Sandmann G. 1996. Expression, purification and properties of lycopene cyclase from *Erwinia uredovora*. *Biochemical Journal* **315,** 869–874.

Suzuki S, Nishihara M, Nakatsuka T, Misawa N, Ogiwara I, Yamamura S. 2007. Flower color alteration in *Lotus japonicus* by modification of the carotenoid biosynthetic pathway. *Plant Cell Reports* **26,** 951–959.

Szabo I, Bergantino E, Giacometti GM. 2005. Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation. *EMBO Reports* **6,** 629–634.

Tanaka Y, Sasaki N, Ohmiya A. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *The Plant Journal* **54,** 733–749.

Tian L, Magallanes-Lundback M, Musetti V, DellaPenna D. 2003. Functional analysis of beta- and epsilon-ring carotenoid hydroxylases in Arabidopsis. *The Plant Cell* **15,** 1320–1332.

Tracewell CA, Cua A, Stewart DH, Bocian DF, Brudvig GW. 2001. Characterization of carotenoid and chlorophyll photooxidation in photosystem II. *Biochemistry* **40,** 193–203.

Veda S, Platel K, Srinivasan K. 2007. Varietal differences in the bioaccessibility of beta-carotene from mango (*Mangifera indica*) and papaya (*Carica papaya*) fruits. *Journal of Agricultural and Food Chemistry* **55,** 7931–7935.

Yano M, Kato M, Ikoma Y, Kawasaki A, Fukazawa Y, Sugiura M, Matsumoto H, Oohara Y, Nagao A, Ogawa K. 2005. Quantitation of carotenoids in raw and processed fruits in Japan. *Food Science and Technology Research* **11,** 13–18.

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287,** 303–305.

Zhou CH, Xu CJ, Sun CD, Li X, Chen KS. 2007. Carotenoids in white- and red-fleshed loquat fruits. *Journal of Agricultural and Food Chemistry* **55,** 7822–7830.