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RESEARCH PAPER

Isolation and functional characterization of a lycopene β-cyclase gene that controls fruit colour of papaya (*Carica papaya* L.)

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Abstract

The colour of papaya fruit flesh is determined largely by the presence of carotenoid pigments. Red-fleshed papaya fruit contain lycopene, whilst this pigment is absent from yellow-fleshed fruit. The conversion of lycopene (red) to β-carotene (yellow) is catalysed by lycopene β-cyclase. This present study describes the cloning and functional characterization of two different genes encoding lycopene β-cyclases (*lcy*-β1 and *lcy*-β2) from red (Tainung) and yellow (Hybrid 1B) papaya cultivars. A mutation in the *lcy*-β2 gene, which inactivates enzyme activity, controls lycopene production in fruit and is responsible for the difference in carotenoid production between red and yellow-fleshed papaya fruit. The expression level of both *lcy*-β1 and *lcy*-β2 genes is similar and low in leaves, but *lcy*-β2 expression increases markedly in ripe fruit. Isolation of the *lcy*-β2 gene from papaya, that is preferentially expressed in fruit and is correlated with fruit colour, will facilitate marker-assisted breeding for fruit colour in papaya and should create possibilities for metabolic engineering of carotenoid production in papaya fruit to alter both colour and nutritional properties.

Key words: Carotenoid, lycopene, lycopene β-cyclase, papaya fruit.

Introduction

Papaya (*Carica papaya* L.) is a fruit crop widely grown in tropical and sub-tropical environments. There has recently been increased interest in the study of the genome of papaya due to its small genome size of 372 Mb and its short life cycle compared with many other tropical fruit tree crops. The two major papaya fruit flesh colours, red and yellow, are controlled by a single genetic locus with yellow being dominant over red (Storey, 1969). The fruit flesh colour of papaya is determined largely by the carotenoid content. Red-fleshed papaya fruit contain high levels of lycopene, whereas yellow-fleshed fruit do not (Yamamoto, 1964; Chandrika *et al.*, 2003). A draft genome sequence of a transgenic (red) papaya has recently been published (Ming *et al.*, 2008).

Carotenoid pigments are indispensable for photosynthesis in plants. In many species, carotenoids can also accumulate as secondary metabolites in chromoplasts of flowers, fruits, seeds or roots to provide distinct coloration, ranging from yellow to orange and red. The carotenoid biosynthesis pathway has been extensively studied in many photosynthetic and non-photosynthetic organisms. Carotenoid biosynthesis begins with the formation of phytoene from geranylgeranyl diphosphate (Fig. 1). Lycopene is a precursor of β-carotene and β-cryptoxanthin. In tomato, lycopene accumulation during fruit ripening is due to the down-regulation of lycopene β-cyclase activity (Pecker *et al.*, 1996). Tomato contains two lycopene β-cyclase genes, including one that is expressed in photosynthetic tissues

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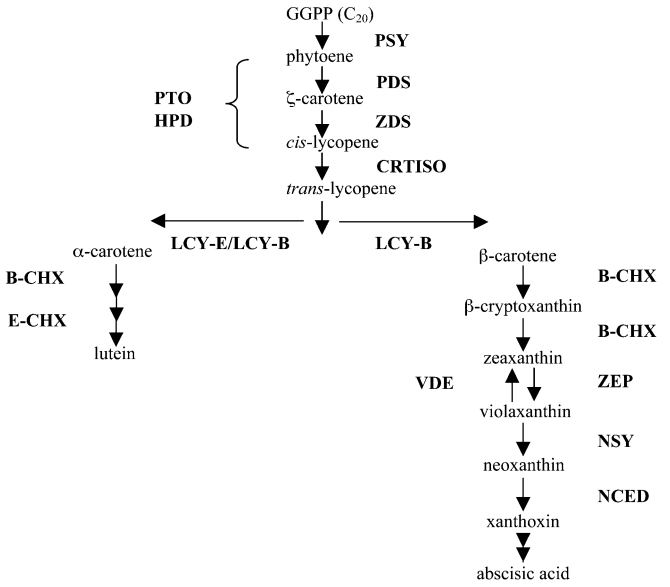


Fig. 1. Carotenoid pathway in plants. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; HPD, 4-hydroxy phenylpyruvate dioxygenase; PTO, plastid terminal oxidase; CRTISO, carotene *cis-trans*-isomerase; LCY-E, lycopene-ϵ-cyclase; LCY-B, lycopene β-cyclase; B-CHX, β-carotene hydroxylase; E-CHX, ϵ-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

(chloroplast-specific) and one that is expressed predominantly in the fruit and flowers (chromoplast-specific). It was expected that a similar situation may occur in papaya. However, Skelton *et al.* (2006) were only able to find a single lycopene β-cyclase gene in papaya and its expression profile did not appear to correlate with lycopene production in the fruit. Furthermore, the sequence of this *lcy*-β gene from both red- and yellow-fleshed papaya cultivars was identical.

Although Skelton *et al.* (2006) identified only one lycopene β-cyclase gene, it was hypothesized that papaya would contain a second such gene, similar to the situation in tomato. In Australia, yellow papaya (known locally as paw-paws) are grown more commonly than red papaya. However, germplasm used in papaya breeding in Australia includes both yellow and red cultivars. Tainung (red) and Hybrid 1B (yellow) are two papaya cultivars grown commercially in Australia that differ in fruit colour (Fig. 2) and flavour. The objectives of this project were to clone all lycopene β-cyclase genes present in red and yellow papaya cultivars and to identify the genetic basis for lycopene production in papaya fruit.

Materials and methods

Plant material

Leaves, flowers, and fruit from various papaya cultivars were obtained from Queensland Primary Industries and Fisheries, Centre for Tropical Agriculture, Southedge, QLD Australia.

Fig. 2. Ripe fruit of the red Tainung (left) and yellow Hybrid 1B (right).

DNA isolation

Genomic DNA was isolated from papaya leaves using a DNeasy Plant kit (Qiagen) according to the manufacturer's instructions.

Design of degenerate primers

An alignment of amino acid sequences of lycopene ϵ-cyclase from tomato, wild tomato, and *Arabidopsis*; lycopene β-cyclase from tomato and capsanthin-capsorubin synthase from bell pepper (Ronen *et al.*, 1999) was used to identify amino acid regions conserved across these enzymes. Two degenerate forward-primers (NNYGVWEDE-F, NNYGVWVDE-F) and two degenerate reverse-primers (PTFLYAMP1-R, PTFLYAMP2-R) were designed to encode the conserved peptide sequences (Table 1).

PCR conditions

PCR amplifications using degenerate primers were done in 25 µl reactions consisting of 0.2 mM dNTPs, 2 mM $MgSO_4$, 0.4 mM of both forward and reverse primers, 0.5 U of Platinum *Taq* polymerase (Invitrogen) and 200 ng of genomic DNA as template. Thermocycler conditions consisted of an initial 3 min denaturation at 95 °C, followed by 30 cycles of 95 °C for 20 s, 48 °C for 30 s, and 68 °C for 60 s.

Cloning of PCR products

PCR amplification products were size-separated on a 1.5% agarose/TAE gel containing 0.1 µl ml^{-1} Gel Red™ fluorescent nucleic acid dye (Biotium), excised and purified using the DNA Clean & Concentrator™ -5 kit (Zymo Research) prior to ligation into the pCR®4-TOPO® vector (Invitrogen).

DNA sequencing

Purified plasmid DNA was sequenced using universal M13 reverse and T7 sequencing primers. Truncated *lcy*-β2 amplicons were sequenced directly using the primers Cp_LCYb2_ORF_F2 and Cp_LCYb2_ORF_R1 (Table 1). Sequencing was performed by the Australian Genome Research Facility (Brisbane, Australia).

RNA extraction

Total RNA for cDNA synthesis was extracted with TRIzol® (Invitrogen) using a modified protocol described elsewhere (Devitt *et al.*, 2006).

5′- and 3′- rapid amplification of cDNA ends

The gene-specific primers Cp_Cycb_F2 and Cp_Cycb_R2 (Table 1) for 5′- and 3′- rapid amplification of cDNA ends (RACE) were

Table 1. Primer sequences used for PCR amplification, SNP detection, and measuring gene expression

Experiment	Primer name	Primer sequence 5′-3′
Lycopene cyclase	NNYGVWEDE-F	AA(T,C)AA(T,C)TA(T,C)GGIGTITGGGA(A,G)GA(T,C)GA
degenerate PCR	NNYGVWVDE-F	AA(T,C)AA(T,C)TA(T,C)GGIGTITGGGTIGA(T,C)GA
	PTFLYAMP1-R	GGCATIGC(A,G)TA(T,C)AA(A,G)AAIGTIGG
	PTFLYAMP2-R	GGCATIGC(A,G)TAIAG(A,G)AAIGTIGG
Lycopene cyclase	Cp_Cycb_F2	AATGGGGTTAAATTCCTCAA
5′ and 3′-RACE	Cp_Cycb_R2	TCCTCCAATACCCTCTTCAC
Lycopene cyclase	Cp_Cycb_F1_5′Racing	AAACAATCGTCAAGCCCTAG
RCA-RACE	Cp_Cycb_R2_3′Racing	GTGAAGAGGGTATTGGAGGA
Lycopene cyclase	Cp_LCYb2_ORF_F2	GCTAGTAAGTTCGGTAACTTCCTTGA
Direct sequencing	Cp_LCYb2_ORF_R1	ATATAGTTTATAATTGCAATCCCTCA
iPLEX SNP	Lcy-β1 primer 1	ACGTTGGATGGGGATCCTACTGTTTCTCTC
assay	Lcy-β1 primer 2	ACGTTGGATGGGTCTTTATGGACTGGAGAG
	Lcy-β1 extension primer	GCATCTTAACAGCAACA
	Lcy-β2 primer 1	ACGTTGGATGTTCTTGCGTCGACCTTATGG
	Lcy-β2 primer 2	ACGTTGGATGCCCCATTGACAACGCAATTC
	Lcy-β2 extension primer	CGTCTTCAGTTTCTTCCTAC
qPCR	Lcy-b2_papaya_qPCR_F	CAGATGCGATTGCGGAGTGC
	Lcy-b2_papaya_qPCR_R	TGGCCTACCCCTGATCATTCTTGT
	Lcy-b1_papaya_qPCR_F	TGGCTATATGGTGGCACGAACTCT
	Lcy-b1_papaya_qPCR_R	CAAGGAACCGAACAATGGAATCTG
	18S_rRNA_papaya_qPCR_F	CTCCGGCGTTGTTACTTTGAAGAA
	18S_rRNA_papaya_qPCR_R	CCCGAAGGCCAACAGAATAGGA

designed based on the degenerate primer-amplified *lcy*-β2 sequence. Total RNA was isolated from ripe fruit flesh of cultivar Hybrid 1B. The 5′- and 3′-RACE were performed using SMART RACE cDNA Amplification Kit (Clontech) and Platinum *Taq* DNA polymerase (Invitrogen) in accordance with the manufacturers' protocols. PCR conditions in the GeneAmp® PCR System 9700 (Applied Biosystems) were as follows: initial denaturation at 94 °C for 3 min followed by 30 cycles with 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 4 min. The 5′- and 3′-RACE products were cloned into pCR4-TOPO (Invitrogen) and transformed into *E. coli* TOP10 cells. Cloned RACE products were fully sequenced in both directions.

RCA-RACE

The rolling circle amplification (RCA)-RACE method of Polidoros *et al.* (2006) was also used to isolate the 5′- and 3′-ends of the lycopene β-cyclase 2 cDNA from papaya. Total RNA from fruit of hybrid 1B and Tainung was used for cDNA synthesis. First-strand cDNA was synthesized using 10 μg of RNA as template and a 5′-phosphorylated oligo(dT)$_{20}$ as primer. After first-strand cDNA synthesis the RNA strand was removed with RNaseH (Invitrogen) and the cDNA was purified using the DNA Clean & Concentrator™-5 purification kit (Zymo Research). cDNA was eluted and circularized using 150 U of Circligase, 50 μM ATP and 1× reaction buffer (Epicentre Biotechnologies) at 60 °C for 1 h, followed by heat inactivation of the enzyme at 80 °C for 10 min. Linear cDNA was removed by the addition of 20 U of Exonuclease1 and Exonuclease reaction buffer (New England Biolabs) and incubation at 37 °C for 45 min. Circular cDNA was purified using the DNA Clean & Concentrator™-5 purification kit and eluted in 6 μl of nuclease free water. An RCA method using the Illustra™ TempliPhi™ amplification kit was then performed in order to amplify the circular cDNA pool. RCA products were used as template to PCR-amplify the full-length papaya *lcy*-β2 gene using primers Cp_Cycb_F1_5′Racing and Cp_Cycb_R2_3′⋆ Racing (Table 1).

Phylogenetic analysis

Amino acid sequences of various lycopene β- and ε-cyclase genes were obtained from GenBank. Multiple alignments of amino acid sequences from papaya LCY-B1 (ABD91578), papaya LCY-B2 (FJ839871), *Arabidopsis* LCY-B (AAF02819), *Arabidopsis* LCY-E (AAF82389), tomato LCY-E (CAA74745), tomato LCY-B1 (CAA60170), and tomato LCY-B2 (AAG21133) were generated using ClustalW version 2.0.11 (Larkin *et al.*, 2007) using BLOSUM as the cost matrix and gap penalties of 10 and 0.1 for opening and extending gaps, respectively. A phylogenetic tree was constructed using MEGA 4 (Tamura *et al.*, 2007). The tree was inferred using the Neighbor–Joining methodology and tested by bootstrapping with 1000 replications. Distances were determined using the p-distance option. The values displayed at the tree nodes are percentage consensus support as determined by the bootstrapping.

Functional validation of expression of papaya lycopene β-cyclases

Full-length or truncated versions of the LCY-B2 cDNA clones isolated from Tainung and Hybrid 1B were cloned into pCRII-TOPO (Invitrogen) and used to transform *E. coli* strain MG1655 P_{T5}-*dxs* P_{T5}-*idi* P_{T5}-*ispDispF* (Yuan *et al.*, 2006) containing pAC-LYC. Plasmid pAC-LYC is a pACYC184 derived vector containing functional carotenoid biosynthesis genes for geranylgeranyl pyrophosphate synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*) from *Erwinia herbicola* (Cunningham *et al.*, 1994). *E. coli* colonies containing pAC-LYC accumulate lycopene and appear pink.

The double transformants were plated onto LB agar supplemented with ampicillin (100 μg ml^{-1}) and chloramphenicol (50 μg ml^{-1}). Colonies were incubated overnight at 37 °C followed by 3 d incubation at room temperature in darkness to maximize carotenoid accumulation.

HPLC profiling of carotenoids in E. coli

E. coli containing LYC-B2 clones were grown in 30 ml of LB media in the dark for 24 h at 37 °C. Carotenoid extraction and

HPLC analysis methods were adapted from Howe and Tanumihardjo (2006). Cells were sedimented by centrifugation and 6 ml of ethanol (containing 0.1% butylated hydroxyl toluene [BHT]) was added to the *E. coli* pellet. After vortexing for 20 s, the solution was extracted five times with 3 ml hexane and then centrifugation was used to separate the phases (5000 *g* for 3 min at 4 °C). Hexane fractions were combined, dried in a centrifugal evaporator at 30 °C, reconstituted in 2 ml methanol/dichloromethane (50/50, v/v), containing 0.1% BHT, filtered through a 0.22 μm syringe filter (Grace) into HPLC vials, and stored under nitrogen at -80 °C prior to HPLC analysis.

The HPLC system consisted of a SIL-10AD VP auto injector (Shimadzu), SCL-10A VP system controller (Shimadzu), LC-10AT VP liquid chromatograph (Shimadzu) and a SPD-M10 A VP diode array detector (Shimadzu). Forty microlitres of each extract was injected onto a YMC C30 Carotenoid Column, 3 μm, 4.6×250 mm (Waters), with a mobile phase consisting of 92% methanol/8% 10 mM ammonium acetate (phase A), and 100% methyl tert butyl ether (phase B). A gradient was used: 0–25 min, 45–5% phase A; 25–27 min, 5% phase A; 27–28 min, 5–45% phase A; 28–33 min, 45% phase A. Lycopene and β-carotene were identified using known standards (Sigma-Aldrich), which showed co-elution and similar absorption spectra.

Quantitative real-time RT-PCR (qPCR)

qPCR was done in a RotorGene RG-3000 instrument (Corbett Life Science) to determine relative *lcy*-β1 and *lcy*-β2 gene expression levels in various organs and fruit development stages. PCR primers were designed from available *lcy*-β1, *lcy*-β2, and 18s rRNA sequences (see Table 1) using Primer Express software v1.5 (Applied Biosystems). The Power SYBR Green RNA-to-C_t 1-step kit (Applied Biosystems) was used for all qPCR experiments. The reverse-transcriptase reaction was done on total RNA from each sample at 48 °C for 30 min followed by a 10 min denaturation at 95 °C to activate the DNA polymerase. The amplification was carried out using the following cycling parameters: 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Fluorescence was acquired at 60 °C. A melt curve from 55–99 °C was performed following each experiment to ensure reactions amplified a single product. Each qPCR experiment was repeated three times and the average results of those reactions reported. Two controls were included in each experiment where either no template or no reverse transcriptase was added.

PCR efficiency determination and estimation of starting concentration

Non-baseline-corrected PCR data were exported from the Rotor-Gene RG-3000 instrument for use in the analysis. Baseline correction and the average PCR efficiency for each of the three genes was determined using the algorithms developed by Ruijter *et al.* (2009) contained within version 11.0 of the qPCR analysis program, LinRegPCR (Ramakers *et al.*, 2003). The starting concentration (N_O) of mRNA from each gene in each tissue sample was calculated using values provided in the LinRegPCR output file by division of the fluorescence threshold (N_t) by the amplication efficiency (E) raised to the power of the normalized fluorescence cycle threshold (ΔC_t):

$$N_O = N_t / E^{\Delta Ct}$$

Cycle thresholds for each gene in each tissue were normalized by deducting the C_t of 18S rRNA from the cycle threshold of the sample of interest. N_O is expressed in arbitrary fluorescence units. Relative gene expression is calculated by directly dividing the N_O of a sample of interest by the N_O of a reference sample.

Single nucleotide polymorphism (SNP) assays

iPLEX assays (Sequenom) were used to identify *lcy*-β1 and *lcy*-β2 alleles present in various red- and yellow-fruited papaya cultivars. Primers used for PCR amplification and extension are listed in Table 1.

Results

A DNA fragment of ~500 bp was PCR-amplified from Hybrid 1B and Tainung genomic DNA using degenerate primers designed from regions conserved across lycopene β- and ε-cyclases and capsanthin-capsorubin synthase. Sequencing of 16 cloned DNA fragments from each cultivar revealed two different, but related, gene sequences from both cultivars: *lcy*-β1 and a second putative lycopene β-cyclase, designated *lcy*-β2.

In order to clone a full-length expressed gene sequence of the newly identified *lcy*-β2, 5′- and 3′-RACE was performed using a combination of two methods: SMART-RACE and RCA-RACE. RCA-RACE amplification using primers designed from sequence discovered using the SMART RACE cDNA amplification method successfully identified full-length *lcy*-β2 sequences from both Tainung and Hybrid 1B. The full length *lcy*-β2 sequence from Hybrid 1B (GenBank accession FJ839871) contained an open-reading frame encoding 500 amino acids which shared 52% sequence identity with LCY-B1 and contained a predicted plastid transit peptide of 71 amino acids. Alignment of the papaya lycopene β-cyclase amino acid sequences with tomato and arabidopsis lycopene β- and ε-cyclases, and subsequent phylogenetic analysis (Fig. 3), revealed that papaya *lcy*-β2 was most similar to the chromoplast-specific lycopene β-cyclase gene from tomato.

To determine the relative expression levels of *lcy*-β1 and *lcy*-β2 genes in red and yellow papaya cultivars the relative mRNA levels of both genes were measured in Tainung and Hybrid 1B leaves, flowers and fruit using qPCR (Fig. 4). The expression level of *lcy*-β2 was generally higher than that of *lcy*-β1. However, the major difference between *lcy*-β1 and *lcy*-β2 was a large increase in *lcy*-β2 gene expression in Hybrid 1B fruit relative to the other tissues. The *lcy*-β2 transcript levels in Hybrid 1B were 15-fold higher in colour-break fruit and 60-fold higher in ripe fruit than in leaves. The *lcy*-β2 transcript levels were 18-fold higher than *lcy*-β1 levels in ripe Hybrid 1B fruit. Such a significant difference in *lcy*-β1 and *lcy*-β2 transcript levels between fruit and

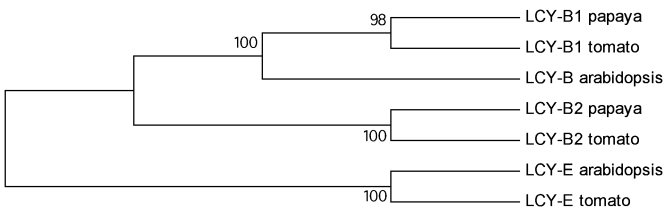
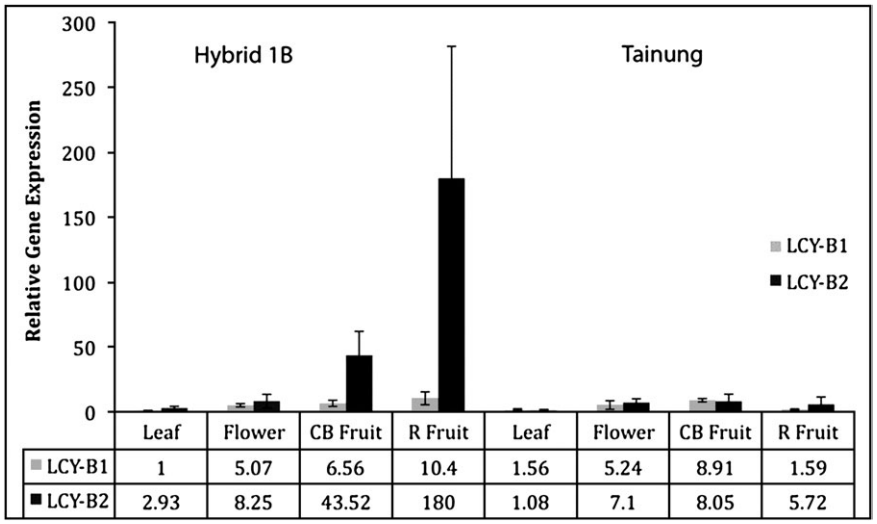


Fig. 3. Phylogenetic tree based on amino acid sequences of lycopene cyclase enzymes. Lycopene ε-cyclase from tomato (CAA74745) and *Arabidopsis* (AAF82389) were compared with *Arabidopsis* lycopene β-cyclase (AAF02819), lycopene β-cyclase 1 and 2 from tomato (CAA60170 and AAG21133), and papaya (ABD91578 and FJ839871).



	Leaf	Flower	CB Fruit	R Fruit	Leaf	Flower	CB Fruit	R Fruit
LCY-B1	1	5.07	6.56	10.4	1.56	5.24	8.91	1.59
LCY-B2	2.93	8.25	43.52	180	1.08	7.1	8.05	5.72

Fig. 4. Expression of *lcy*-β1 and *lcy*-β2 in various tissues of papaya Hybrid 1B and Tainung. Relative levels of *lcy*-β1 and *lcy*-β2 mRNA from leaf, flower, colour-break (CB) fruit, and ripe (R) fruit of cultivars Hybrid 1B and Tainung were measured by qPCR using gene-specific primers. Expression data were normalized to the expression of 18S rRNA. Expression levels in each tissue are relative to the expression of *lcy*-β1 in leaf tissue of Hybrid 1B.

leaves was not found in Tainung, where there was a relatively small increase in *lcy*-β2 gene expression in fruit compared with leaf (Fig. 4).

In order to demonstrate function of *lcy*-β2 from Tainung and Hybrid 1B, each gene was cloned into pCRII-TOPO and transformed into *E. coli* harbouring the plasmid pAC-LYC, which contains genes enabling lycopene biosynthesis. Each gene was cloned as either a full-length, or a truncated sequence lacking the predicted chloroplast transit peptide. *Lcy*-β2 from Hybrid 1B was functional in that truncated clones were able to convert lycopene to β-carotene, but the gene from Tainung was not functional in either full-length or truncated clones (Table 2). β-carotene was undetectable by HPLC in Tainung, while 70% of the available lycopene was converted to β-carotene in Hybrid 1B. These results suggest that a non-functional *lcy*-β2 may be responsible for the accumulation of lycopene in the red Tainung fruit.

Comparison of the nucleotide sequences of *lcy*-β2 from Hybrid 1B and Tainung revealed two polymorphisms, both in the coding region of the gene. At nucleotide 607, Hybrid 1B contained a cytosine (C) that was substituted for an adenosine (A) in Tainung. Starting at position 881 Hybrid 1B has a run of five thymidines (T), whereas Tainung had an insertion of an additional two Ts. The A/C sequence polymorphism leads to a single amino acid change, whereas the TT/- mutation leads to a frame-shift and premature translation termination. To determine whether either of these polymorphisms was correlated with the production of red versus yellow fruit colour, SNP and sequencing assays were done on 12 colour-differentiated cultivars. Genotyping using an iPLEX SNP assay for the A/C polymorphism at position 607 showed that all papaya cultivars with A at this position produced red fruit, except for Kapoho, which is yellow (Table 3). The remaining yellow-fruit cultivars tested contained either C or a heterozygous A/C polymorphism at this position. PCR amplification and sequencing of the *lcy*-β2 gene from Kapoho confirmed that it contained an adenosine at position 607, but it lacked the TT insertion at position 881 (Table 3). Both full-length and truncated *lcy*-β2 clones from Kapoho were used to transform a lycopene-producing strain of *E. coli*, which confirmed that it contained a functional *lcy*-β2 gene able to convert lycopene to β-carotene (data not shown). Therefore, it could be

Table 2. Expression of Papaya lycopene β-cyclase genes in *E. coli* analysed by HPLC profiling

Papaya lycopene β-cyclase gene	Lycopene (%)	β-carotene (%)
None (pAC-LYC only)	100	0
Hybrid 1B lcy-β2	50	50
Hybrid 1B lcy-β2 (without transit peptide)	30	70
Tainung lcy-β2	100	0
Tainung lcy-β2 (without transit peptide)	100	0

Table 3. Combined results of genotyping and sequencing of *lcy*-β2 alleles across various papaya cultivars

Cultivar	Sequence (607)	SNP (607)	Insertion (881)	Phenotype
Hybrid 1B	C	C,A	–	Yellow
BB9H	C	N.D.	–	Yellow
Kapoho	A	A	–	Yellow
Peter Gray	C	C	–	Yellow
Tainung	A	A	TT	Red
Maradol Roja	A	A	TT	Red
Paris	A	A	TT	Red
Eksotica	A	A	TT	Red
Mission Beach Red	A	A	TT	Red
Sunrise Solo	A	A	TT	Red
Subang	A	A	TT	Red

concluded that the TT insertion at 881 is responsible for the inactivation of *lcy*-β2 in papaya and not the A/C polymorphism at position 607 in the gene sequence. Sequencing of *lcy*-β2 from a number of yellow- and red-fleshed papaya cultivars confirmed that all of the red cultivars contained the TT insertion at position 881 (Table 3).

Discussion

Our attempt to isolate all of the lycopene cyclase genes from papaya yielded two different lycopene β-cyclases. One of these genes had previously been cloned and characterized by Skelton *et al.* (1996), but the second lycopene β-cyclase is new. The newly discovered lycopene β-cyclase gene (*lcy*-β2) is more closely related to chromoplast-specific β-cyclases from other species than to the papaya *lcy*-β1 gene, suggesting that it is also a chromoplast-specific gene and may play a significant role in carotenoid production and control of fruit colour in papaya. This was supported by gene expression studies which showed that *lcy*-β2 is much more highly expressed in ripe fruit than in leaves.

The functional analysis of LCY-B2 activity across a number of papaya genotypes was conducted using *E. coli* engineered for enhanced carotenoid substrate accumulation. The identification of a functional LCY-B2 in the yellow-fleshed Kapoho variety was particularly helpful in challenging our early hypothesis that the A/C sequence polymorphism at position 607 and resulting amino acid change may have been responsible for the reduced enzyme functionality in red-fleshed varieties.

The position 607 SNP assay identified a correlation between SNP and red flesh colour in all genotypes tested except Kapoho. Complete sequencing of multiple *lcy*-β2 clones from all varieties across both DNA strands successfully identified the TT insertion at position 881 in all red-fleshed varieties. From the combination of all these data it was concluded that *lcy*-β2 encodes a chromoplast-specific lycopene β-cyclase gene, which controls papaya fruit colour. A TT insertion in the *lcy*-β2 gene results in an inactive chromoplast-specific lycopene β-cyclase and leads to the accumulation of lycopene in the fruit of red-fleshed varieties.

Over the the past 10 years, a number of studies of carotenogenic fruit have identified the key importance of lycopene cyclase activity in determining the profile of fruit carotenoids. Studies of carotenoid mutants of tomato by Ronen *et al.* (2000) led to the map-based cloning of a second, chromoplast-specific, lycopene β-cyclase and a new model of alternative carotenoid regulation in chromoplasts and chloroplasts in tomato.

The presence of two lycopene β-cyclases in papaya is similar in some respects to a number of other species that accumulate lycopene in their fruit. Tomato contains two lycopene β-cyclase genes, *CrtL-b* and *B*, with the *B* gene expressed exclusively in chromoplast-containing tissues of flowers and fruits (Ronen *et al.*, 2000). However, in contrast to the situation in papaya, expression of the tomato *B* gene in fruits is low and limited to a short period around the breaker stage, leading to mostly lycopene and a little β-carotene accumulation in wild-type tomato fruit. Overexpression of a bacterial lycopene β-cyclase in tomato fruit has been shown to increase β-carotene production (Wurbs *et al.*, 2007).

While citrus species do not typically accumulate lycopene in fruit, lycopene-accumulating mutants in grapefruit and orange do exist, and their carotenoid content and carotenoid pathway gene expression have recently been studied. A chromoplast-specific lycopene β-cyclase (*Csβ-LCY2*) has been characterized from *Citrus sinensis* (cv. Navel) and the lycopene-accumulating mutant, *Citrus paradise* (cv. Star Ruby) (Alquézar *et al.*, 2009). In Navel oranges, *Csβ-LCY2* was highly expressed during fruit maturation in contrast to *Csβ-LCY1*, which displayed a low, unchanging expression pattern during fruit maturation. Two alleles of the *LCY2* gene were isolated and 16 amino acid changes were proposed to account for the difference between the functional *β-LCY2a* allele from Navel orange and the almost inactive allele, *β-LCY2b*, from Star Ruby grapefruit.

The presence of two lycopene β-cyclase genes can account for the observed carotenoid accumulation in red and yellow papaya fruit. In yellow fruit, both *lcy*-β1 and *lcy*-β2 are expressed, although the *lcy*-β2 gene is expressed at a much higher level and accounts for 95% of all β-cyclase mRNA. The level of β-cyclase enzyme activity resulting from the expression of β1 and β2 is sufficient to convert all of the available lycopene to β-carotene (and subsequently to β-cryptoxanthin). However, in red papaya fruit, the β2 transcript level is reduced and the gene product is non-functional. Therefore, the only β-cyclase enzyme activity would be from the *lcy-β1* gene. This *lcy*-β1 activity is not sufficient to convert all of the lycopene to β-carotene, resulting in accumulation of both lycopene and β-carotene in red-fleshed papaya fruit.

The isolation and characterization of the two lycopene β-cyclase genes from papaya has potential applications to enhance papaya breeding and genetic improvement. The most obvious use of this information is for the early selection of fruit colour in papaya breeding through the development of a molecular marker assay to target the TT insertion in *lcy*-β2. Further modification and selection of carotenoid composition of papaya may also be possible through the up- or down-regulation of β-cyclases and other genes in the carotenoid pathway.

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