

RESEARCH PAPER

Plastid structure and carotenogenic gene expression in red- and white-fleshed loquat (*Eriobotrya japonica*) fruits

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Abstract

Loquat (*Eriobotrya japonica* Lindl.) can be sorted into red- and white-fleshed cultivars. The flesh of Luoyangqing (LYQ, red-fleshed) appears red-orange because of a high content of carotenoids while the flesh of Baisha (BS, white-fleshed) appears ivory white due to a lack of carotenoid accumulation. The carotenoid content in the peel and flesh of LYQ was approximately 68 µg g⁻¹ and 13 µg g⁻¹ fresh weight (FW), respectively, and for BS 19 µg g⁻¹ and 0.27 µg g⁻¹ FW. The mRNA levels of 15 carotenogenesis-related genes were analysed during fruit development and ripening. After the breaker stage (S4), the mRNA levels of phytoene synthase 1 (*PSY1*) and chromoplast-specific lycopene β-cyclase (*CYCB*) were higher in the peel, and *CYCB* and β-carotene hydroxylase (*BCH*) mRNAs were higher in the flesh of LYQ, compared with BS. Plastid morphogenesis during fruit ripening was also studied. The ultrastructure of plastids in the peel of BS changed less than in LYQ during fruit development. Two different chromoplast shapes were observed in the cells of LYQ peel and flesh at the fully ripe stage. Carotenoids were incorporated in the globules in chromoplasts of LYQ and BS peel but were in a crystalline form in the chromoplasts of LYQ flesh. However, no chromoplast structure was found in the cells of fully ripe BS fruit flesh. The mRNA level of plastid lipid-associated protein (*PAP*) in the peel and flesh of LYQ was over five times higher than in BS peel and flesh. In conclusion, the lower carotenoid content in BS fruit was associated with the lower mRNA levels of *PSY1*, *CYCB*, and *BCH*; however, the failure to develop normal chromoplasts in BS flesh is the most convincing explanation for the lack of carotenoid accumulation. The expression of *PAP* was well correlated with chromoplast numbers and carotenoid accumulation, suggesting its possible role in chromoplast biogenesis or interconversion of loquat fruit.

Key words: Carotenoid, chloroplast, chromoplast, colour, gene expression, fruit development, fruit ripening, loquat, plastid.

Introduction

Carotenoids are essential for plants, harvesting light for photosynthesis, protecting plants from high light stress, and furnishing flowers and fruits with bright yellow, red, or orange colors that attract animals and facilitate pollination

Abbreviations: *BCH*, β-carotene hydroxylase; *BS*, Baisha; *CCD*, carotenoid cleavage dioxygenase; *CCl*, citrus colour index; *CPW* 18M, cell and protoplast washing solution with 18% mannitol; *CHRC*, chromoplast-specific carotenoid associated protein; *CRTISO*, carotene isomerase; *CYCB*, chromoplast-specific lycopene β-cyclase; *DAFB*, days after full bloom; *DMAPP*, dimethylallyl diphosphate; *DTT*, DL-dithiothreitol; *DXP*, 1-deoxy-D-xylulose 5-phosphate; *DXR*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *DXS*, 1-deoxy-D-xylulose 5-phosphate synthase; *ECH*, ε-carotene hydroxylase; *FW*, fresh weight; *GAP*, D-glyceraldehyde 3-phosphate; *GGPP*, geranylgeranyl diphosphate; *GGPS*, geranylgeranyl diphosphate synthase; *HMBPP*, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate; *IDI*, isopentenyl pyrophosphate isomerase; *IDS*, isopentenyl pyrophosphate synthase; *IPP*, isopentenyl pyrophosphate; *LCYB*, lycopene β-cyclase; *LCYE*, lycopene ε-cyclase; *LYQ*, Luoyangqing; *NCED*, 9-cis-epoxycarotenoid dioxygenase; *NSY*, neoxanthin synthase; *PAP*, plastid lipid-associated protein; *PDS*, phytoene desaturase; *PSY*, phytoene synthase; *PVP*, polyvinylpyrrolidone; *Q-PCR*, real-time quantitative PCR; *TEM*, transmission electron microscopy; *VDE*, violaxanthin de-epoxidase; *ZDS*, ζ-carotene desaturase; *ZISO*, ζ-carotene isomerase; *ZEP*, zeaxanthin epoxidase.

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and seed dispersal (Howitt and Pogson, 2006). Carotenoids are also important for the nutrition of animals and humans, providing essential nutrients and health-promoting compounds. Beta-carotene, β -cryptoxanthin and α -carotene are the primary dietary sources of vitamin A, and other carotenoids, such as lycopene, have a strong antioxidant function that could reduce the risk of cancer and cardiovascular diseases (Hadley *et al.*, 2002).

The valuable roles of carotenoids have been the subjects of much research and carotenoid biosynthesis in higher plants has been widely studied in the past two decades (Hirschberg, 2001; Bramley, 2002; Fraser and Bramley, 2004; Taylor and Ramsay, 2005; DellaPenna and Pogson, 2006; Lu and Li, 2008). Carotenoids are synthesized from the five-carbon intermediates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In plants, there are two pathways for the synthesis of IPP. One is the mevalonate pathway where IPP and DMAPP are synthesized from mevalonic acid. The other, known as the methylerythritol phosphate (MEP) pathway, occurs in plastids and utilizes glyceraldehyde-3-phosphate (GAP) and pyruvate for IPP and DMAPP synthesis (Fig. 1) (Cordoba *et al.*, 2009). Three IPPs and one DMAPP are condensed to form C20 geranylgeranyl pyrophosphate (GGPP). Two molecules of GGPP are converted to colourless phytoene by a phytoene synthase (PSY). Phytoene is then converted via ζ -carotene to lycopene, catalysed by phytoene desaturase (PDS), ζ -carotene desaturase (ZDS), carotene isomerase (CRTISO), and ζ -carotene isomerase (Z-ISO) (Chen *et al.*, 2010). Subsequently, the ends of the linear carotenoid lycopene can be cyclized by lycopene β -cyclase (LCYB) and lycopene ϵ -cyclase (LCYE) to synthesize α -carotene or cyclized by a lycopene β -cyclase (LCYB)/chromoplast-specific lycopene β -cyclase (CYCB) (Ronen *et al.*, 2000; Alquezar *et al.*, 2009) alone to synthesize β -carotene. Alpha-carotene and β -carotene are hydroxylated to produce lutein and zeaxanthin, respectively, catalysed by β -ring hydroxylase (BCH) and $\epsilon\epsilon$ -ring hydroxylase (ECH). Further, zeaxanthin is transformed into violaxanthin by zeaxanthin epoxidase (ZEP), and is reversed by violaxanthin de-epoxidase (VDE) to give rise to the xanthophyll cycle for plants to adapt to high light stress. Violaxanthin is converted into neoxanthin by neoxanthin synthase (NSY). One important product of the pathway is abscisic acid, the formation of which is catalysed by 9-cis-epoxycarotenoid dioxygenase (NCED).

Different carotenoid patterns are found in different plant species and cultivars, and the mechanisms have been investigated. It is known that lycopene is the typical carotenoid of ripe tomato, which can be explained by the increasing mRNA levels of *PSY1* and *PDS* (Giuliano *et al.*, 1993) and decreasing expression levels of *LCYE* and *LCYB* during ripening (Pecker *et al.*, 1996; Ronen *et al.*, 1999). Capsanthin and capsorubin are the key components of red pepper, due to high expression levels of capsanthin-capsorubin synthase (CCS) genes (Ha *et al.*, 2007). Different carotenoid compositions and levels also underlie colour differences between cultivars. For example, white pepper,

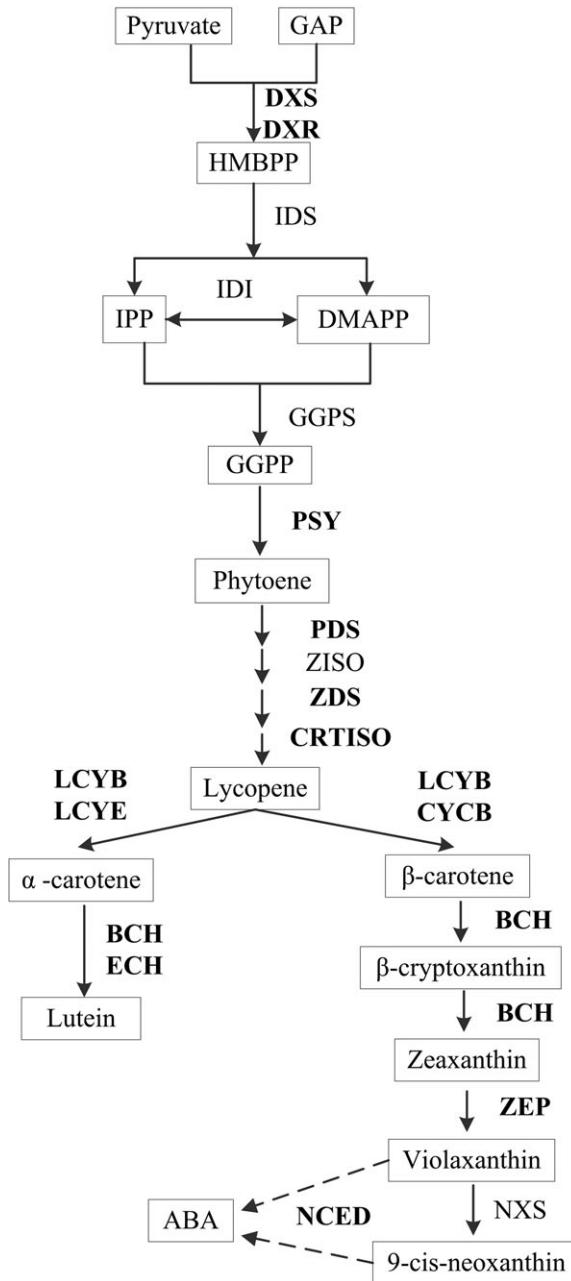


Fig. 1. Carotenoid biosynthetic pathway in higher plants. Genes with expression levels studied are in bold letters. GAP, D-glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate-synthase; DXR, DXP reductoisomerase; HMBPP, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate; IDS, isopentenyl pyrophosphate synthase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; IDI, isopentenyl pyrophosphate isomerase; GGPS, geranylgeranyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; PSY1, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ -carotene desaturase; CRTISO, carotene isomerase; ZISO, ζ -carotene isomerase; LCYE, lycopene ϵ -cyclase; LCYB, lycopene β -cyclase; CYCB, chromoplast-specific lycopene β -cyclase; BCH, β -carotene hydroxylase; ECH, $\epsilon\epsilon$ -carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase.

which lacks carotenoids, has been shown to result from the absence of expression of *PSY*, *PDS*, *BCH*, and *CCS* (Ha *et al.*, 2007). Compared with yellow cultivars, a higher expression level of a carotenoid cleavage dioxygenase (CCD) gene was observed in the white petals of chrysanthemum and the white flesh of peach, where carotenoids are thought to be produced but subsequently degraded into colourless compounds (Ohmiya *et al.*, 2006; Brandi *et al.*, 2011). Yellow and red cultivars of carrot root predominantly contained lutein (74%) and lycopene (57%), respectively, which is consistent with the high expression levels of *LCYE* and *ZDS*, respectively (Cloutault *et al.*, 2008). Different patterns of accumulation of β-carotene resulted in the diverse colour of *Actinidia* (kiwifruit) species, and transcripts of *LCYB* were associated with β-carotene accumulation (Ampomah-Dwamena *et al.*, 2009). Sometimes selective expression of functional alleles could also influence carotenoid accumulation. As an example, red-fleshed Star Ruby grapefruit predominantly expressed the dysfunctional *β-LCY2b* allele during fruit ripening whereas Navel oranges preferably expressed the functional allele *β-LCY2a* (Alquezar *et al.*, 2009).

Transcriptional regulation of the carotenoid biosynthesis pathway cannot always explain the differential accumulation of carotenoids in plant tissues. Expression levels of the carotenogenic genes in the orange cauliflower mutant (*or*) were similar to those of the wild-type cauliflower (Li *et al.*, 2001). Expression of carotenogenesis-related genes was observed in a white carrot root which does not contain carotenoids (Cloutault *et al.*, 2008). Similarly, major carotenogenic gene expression (*PSY1*, *PDS*, and *ZDS*) cannot explain the contrasting colours of different apricot varieties (*Prunus armeniaca*), and Marty *et al.* (2005) surmise that other types of regulation may operate, including post-transcriptional or metabolic regulation.

Carotenoids are synthesized in plastids, and the differential accumulation of carotenoids has been shown in some cases to be related to plastid biogenesis and interconversion (Egea *et al.*, 2010). In the white carrot cultivar, Kim *et al.* (2010) showed that the chromoplasts contained fewer carotenes and were reduced in number compared with the orange variety. The high β-carotene content in the orange curd of a cauliflower mutant was shown to be due to the differentiation of proplastids and other uncoloured plastids into chromoplasts (Paolillo *et al.*, 2004; Lu *et al.*, 2006). Similarly, *hp1* and *hp2* tomato accumulate high levels of carotenoid, due probably to an increased plastid number and size, which provides a larger compartment for carotenoid biosynthesis and deposition (Liu *et al.*, 2004; Kolotilin *et al.*, 2007).

An increase in the size and number of plastoglobulins is generally observed during the transition of chloroplasts to chromoplasts and it is believed that they participate in carotenoid sequestration (Bréhélin and Kessler, 2008; Egea *et al.*, 2010). In tomato, suppression of the chromoplast-specific carotenoid associated protein (CHRC) expression by RNAi leads to a 30% reduction in carotenoid content in tomato flowers (Leitner-Dagan *et al.*, 2006). Numerous CHRC/Fib homologues have been collectively termed

plastid lipid-associated proteins (PAPs; Leitner-Dagan *et al.*, 2006).

Loquat (*Eriobotrya japonica* Lindl.), a member of the Rosaceae, accumulates carotenoids as the main pigments in mature fruit. The fruit can be divided into two groups, red- and white-fleshed, according to the colour of the flesh, and our previous study observed the differential accumulation of carotenoids in the fruits of these two groups (Zhou *et al.*, 2007). The goal of the present study was to investigate the mechanism leading to the diversity of carotenoid patterns in Luoyangqing (LYQ, red-fleshed) and Baisha (BS, white-fleshed) cultivars. The carotenoid content of peel and flesh was monitored during the development of LYQ and BS, using chemical measurements and microscopy. The mRNA levels of 14 carotenogenic genes, indicated in Fig. 1 in bold letters, as well as the *Or* gene were analysed during the development and ripening of LYQ and BS fruits. In addition, expression of *PAP* was also measured in fruit at the fully ripe stage. The results suggested that the differential accumulation of carotenoids between cultivars was associated with differential expression of *PSY1*, *CYCB*, and *BCH*. However, microscopic and ultrastructural data indicate that failure to develop normal chromoplast structures was more critically responsible for the lack of carotenoid accumulation in BS flesh.

Materials and methods

Plant materials

Luoyangqing (LYQ, red-fleshed) and Baisha (BS, white-fleshed) loquats (*Eriobotrya japonica* Lindl.) were sampled from an orchard in Luqiao, Zhejiang, China. Fruit of six developmental stages, S1, fruitlet, 45 d after full bloom (DAFB); S2, immature green, 75 DAFB; S3, mature green, 95 DAFB; S4, breaker, 105 DAFB; S5, half ripe, 110 DAFB; S6, fully ripe, 115 DAFB, were collected (Fig. 2). From these fruits, except those of S1 and S2, the peel was separated from the rest of the fruit (flesh) and both tissues were immediately frozen in liquid nitrogen, then powdered, and carotenoids and RNA were extracted from aliquots of the same samples.

Colour measurement

Peel colour at different developmental stages was measured using a Hunter Lab Mini Scan XE Plus colorimeter (Hunter Associates Laboratory, Inc., USA). The CIE $L^*a^*b^*$ colour scale was adopted, and the data were expressed as L^* , a^* , b^* , C^* , H^* , as well as the citrus colour index (CCI). The CCI is a comprehensive indicator for colour impression [$CCI=1000\times a^*/(L^*\times b^*)$] with positive CCI indicating red, negative as blue-green, and zero for an intermediate mixture of red, yellow, and blue-green (Zhou *et al.*, 2010). Four random measurements per fruit were made and a mean value was obtained from six fruits per replicate.

Carotenoid extraction, quantification, and HPLC analysis

Carotenoids were extracted from fruits and analysed by HPLC, according to a method previously described by Xu *et al.* (2006). Approximately 100 mg of peel and 500 mg of flesh were extracted with chloroform/methanol/50 mM TRIS buffer, pH 7.5. The chloroform phase was collected by 10 min centrifugation at 10 000 g and the aqueous phases were re-extracted with chloroform. The two combined chloroform phases were dried under nitrogen gas;



Fig. 2. Photographs showing the colours of LYQ (A) and BS (B) fruits at different stages (S1–S6) of development and ripening used during this study. Bar, 2 cm.

the residue was first dissolved in 20 µl of diethyl ether and then in 6% (w/v) KOH in methanol, incubated at 60 °C for 30 min in darkness to complete saponification. Water and chloroform were then added, and the samples were extracted as described above, and finally dried with nitrogen gas. Three replicates were performed for each sample. HPLC analysis was carried out on Waters Alliance 2695 system (Waters Corporation, USA) consisting of a 2695 module and a 2996 PDA detector, equipped with a 250×4.6 mm i.d., 5 µm, YMC reverse-phase C₃₀ column and a 20×4.6 mm i.d., YMC C₃₀ guard. Chromatography was carried out at 25 °C with the elution programme as previously described by Zhou *et al.* (2007). Compounds were detected at 450 nm (violaxanthin, luteoxanthin, 9-cis-violaxanthin, lutein, β-cryptoxanthin, and β-carotene) or 400 nm (ζ-carotene) or 286 nm (phytoene) or 348 nm (phytofluene) and quantified according to their respective standard curves.

Cloning of cDNAs encoding carotenogenic enzymes

Total RNA was extracted from frozen powder following our previously published protocol (Shan *et al.*, 2008). For the 15 target carotenogenesis-related genes, degenerate primers were designed based on sequences corresponding to highly conserved peptide regions of known gene sequences of other plants from the National Center for Biotechnology Information (NCBI) (see Supplementary Table S1 at JXB online). The sequence of a plastid lipid-associated protein gene (*PAP*) fragment was obtained from another study involving deep sequencing, and the 3'-untranslated regions (UTR) of the candidate sequence was amplified using the SMART™ RACE cDNA amplification Kit (Clontech) with PAPSP (5'-GAAGCTCCT-GACATTGTCAAGAACAA-3') as the gene-specific primer. cDNA

synthesized from LYQ at stage 6 were used as PCR templates. Amplified PCR products of appropriate size were cloned into pMD18-T vector (Takara, China) and were sequenced by Invitrogen (China). Nucleotide homology to genes from other plants was analysed using BLAST programmes from the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Real-time quantitative PCR (Q-PCR) analysis

cDNA was synthesized according to a method previously described by Wang *et al.* (2010). The Q-PCR reactions were performed in a total volume of 20 µl, including 1 µl of each primer (10 µM), 2 µl cDNA, 7 µl PCR-grade water, and 10 µl of 2× Platinum SYBR Green Supermix (Bio-Rad, USA) on an iCycler iQ Q-PCR instrument (Bio-Rad, USA). The PCR programme was initiated with a preliminary step of 5 min at 95 °C, followed by 45 cycles at 95 °C for 5 s, 58 °C for 15 s, and 72 °C for 10 s. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. No-template controls for each primer pair were included in each run. The gene-specific primers for Q-PCR were designed according to the gene sequences obtained in this study, which have been deposited in GenBank with accession numbers JN004208–JN004223, JN204273 (Table 1). The expression level of *actin* was used to normalize the mRNA levels for each sample, with abundance expressed as a multiple of *actin*.

Light microscopy

Protoplasts were prepared as follows: peel and flesh were cut into 2–3 cm² pieces with a sterile scalpel blade. One gram of peel or

Table 1. Primers used for real-time quantitative PCR (Q-PCR)

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)	GenBank accession number
DXS	5'-GGTCATCACTATTGAAGA-3'	5'-TCAAATTGGCCACTCCATG-3'	206	JN004208
DXR	5'-CATCCAACTGGAATATGGG-3'	5'-GCCAGAACAGATGAATCCTG-3'	182	JN004209
PSY1	5'-ACAGATGAGCTAGTGGATGG-3'	5'-CATTCTTCTACCATGTCT-3'	186	JN004210
PDS	5'-AATGAGATGCTGACTTGGCC-3'	5'-TTGGAACCGTGTTCCTG-3'	263	JN004211
ZDS	5'-AAGAAATGCACTGGCTTGC-3'	5'-CCACCTTGGACAAGAACCA-3'	126	JN004212
CRTISO	5'-AGCATTCCAACGTGTTCTGTA-3'	5'-TTCTTGCCCTAGTCCTT-3'	122	JN004213
LCYB	5'-CAAACGGTGTAAATTCACCA-3'	5'-CCACTGGTAACCTGGATTGTA-3'	177	JN004214
CYCB	5'-AACCATGGATATCAGATTGCTC-3'	5'-GAATTACTAGTGCAGCAAATAAGG-3'	134	JN004215
LCYE	5'-ACTAGATTGTTCTTGAGGA-3'	5'-CAACCGGAATCCAAGACCA-3'	148	JN004216
BCH	5'-GAGAAGGTCCCGTTCGAGCT-3'	5'-AGGCCATCATGGACAAACAT-3'	172	JN004217
ECH	5'-CGTTCTCAAAGGTGGGG-3'	5'-TCCAGCAGAGCAACTGATC-3'	155	JN004218
ZEP	5'-TACACTGGATATCGCAGATT-3'	5'-TGAACGCATACCACTGCAT-3'	128	JN004219
VDE	5'-TCTGATGTGGAGAATTCC-3'	5'-TCATGCAATTGGCAATCAA-3'	137	JN004220
CCD	5'-AAGAGCGTGCTGCGAAAT-3'	5'-AGTAGGTTCCGATTACCAT-3'	119	JN004221
Or	5'-GAACGGATATCTTGCTGTGC-3'	5'-GTAGGGCACATTACCTTCC-3'	157	JN004222
Actin	5'-AATGGAACGGAAATGGCAAGGC-3'	5'-TGCCAGATCTCTCCATGTCATCCC-3'	227	JN004223
PAP	5'-CGAGGTTAAACTCCAGAT-3'	5'-GCTGAAGCGTTACATTTACA-3'	156	JN204273

flesh pieces was transferred to 2 ml tubes containing 1.5 ml CPW 18M (cell and protoplast washing solution with 18% mannitol) (pH 5.8) (Frearson *et al.*, 1973) containing 1% cellulase, 0.3% macerozyme, and 0.05% pectolyase. The tubes were incubated at 28 °C on a reciprocating shaker (20–30 rpm) overnight. Protoplasts were washed and held in CPW 18M and observed and photographed with an Olympus light microscope (Japan).

Plastids were isolated from 20 g flesh or 5 g peel for each cultivar as follows: Tissues were homogenized with a Blitzkrieg Extractor (JHBE-50S) in 100 ml of grinding buffer (0.45 M sucrose, 5 mM EDTA, 1 mM DTT, 0.1% BSA, 0.1% PVP, 50 mM TRIS, and 10 mM Tricine, pH 7.5). The homogenate was filtered through four layers of Miracloth. The cellular debris was eliminated by 5 min of centrifugation at 800 g, and the chromoplast fraction was pelleted by 20 min centrifugation at 10 000 g. The pellet was gently resuspended in 2 ml grinding buffer, and observed and photographed with an Olympus light microscope (Japan).

Transmission electron microscopy (TEM)

Small pieces (2–3 mm²) of peel and flesh were cut and fixed for more than 4 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). After fixation, samples were washed three times in the phosphate buffer; then post-fixed with 1% OsO₄ in 0.1 M phosphate buffer (pH 7.0) for 1 h and washed three times in the phosphate buffer. Tissue samples were dehydrated by a graded series of ethanol (50%, 70%, 80%, 90%, 95%, and 100%) for about 15–20 min at each step, and transferred to absolute acetone for 20 min for infiltration. The first infiltration step was carried out with a 1:1 mixture of absolute acetone and the final Spurr resin mixture for 1 h at room temperature. The second step was the transfer to a 1:3 mixture of absolute acetone and the final resin mixture for 3 h. The third step was carried out in the final Spurr resin mixture overnight. After the third infiltration step, embedded samples were placed in capsules contained embedding medium and heated at 70 °C for about 9 h. The samples were stained by uranyl acetate and alkaline lead citrate for 15 min each and observed in TEM using a Hitachi JEM-1230 (Japan).

GenBank accession numbers

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers: JN004208 (DXS),

JN004209 (DXR), JN0042010 (PSY1), JN0042011 (PDS), JN0042012 (ZDS), JN0042013 (CRTISO), JN0042014 (LCYB), JN0042015 (CYCB), JN0042016 (LCYE), JN0042017 (BCH), JN0042018 (ECH), JN0042019 (ZEP), JN0042020 (VDE), JN0042021 (CCD), JN0042022 (Or), JN0042023 (actin), JN204273 (PAP).

Results

Carotenoid content and composition analysis of two loquat cultivars

The peel colour of loquat fruit changed from green to orange (LYQ) or yellow (BS), while the flesh colour turned from yellowish to orange in LYQ, but remained ivory white in BS throughout development and ripening (Fig. 2). The CIE colour values of peels are in good accordance with the visual colour (see *Supplementary Table S3* at *JXB* online).

The differences in carotenoid content and composition of red- and white-fleshed loquats were first analysed for fruits at the fully ripe stage (S6). The total content of carotenoids was much higher in the peel than in the flesh, and in LYQ compared with BS. The main carotenoids accumulated were β-carotene and lutein in peel, and β-carotene as well as β-cryptoxanthin in flesh, with a higher percentage of lutein in BS than LYQ, both in peel and flesh (Fig. 3A).

The changes in carotenoid content and composition during fruit development and ripening were analysed by HPLC in two cultivars. In the present study whole fruits were analysed at the S1 and S2 stages because the peel was hard to separate from the rest of the fruit (flesh) at these stages, but at all later stages the peel was separated from the flesh. The differences in carotenoid content and composition in LYQ and BS were quantified by HPLC (Fig. 3A, B). During the stages S1–S2, the total carotenoid levels in LYQ was slightly higher than in BS, but the carotenoid profiles were almost the same, and mainly comprised the usual chloroplast-type carotenoids, especially lutein (48.6% of total carotenoids

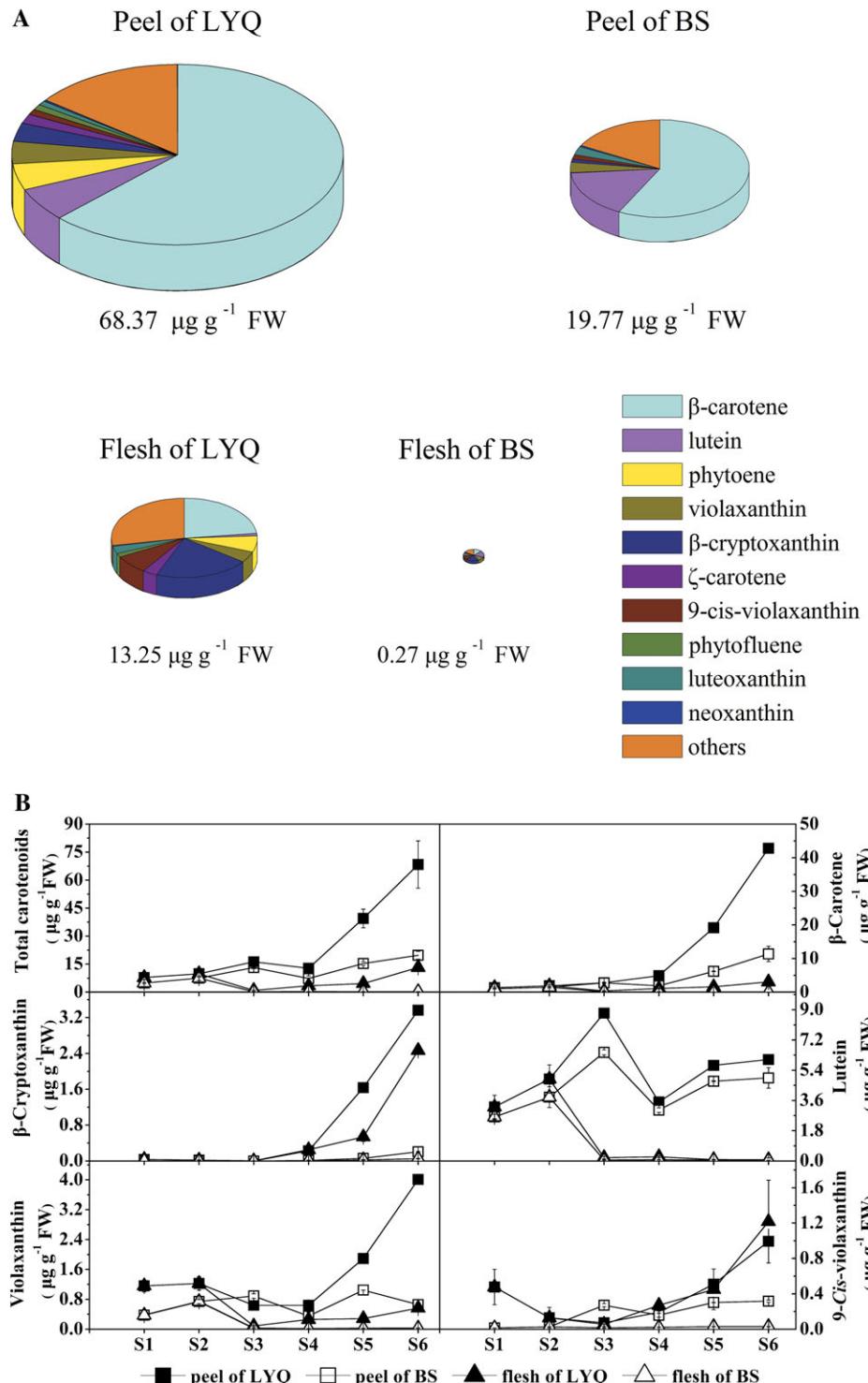


Fig. 3. Carotenoid content and composition in LYQ and BS loquat fruits. (A) Pie chart representation of carotenoid composition in the peel and flesh of mature ripe loquat fruits. (B) Carotenoid contents in the peel and flesh of fruits during development and ripening. Fruit development and ripening was divided into six stages: S1, fruitlet; S2, immature green; S3, mature green; S4, breaker; S5, half-ripe; S6, fully ripe, as shown in Fig. 2. From these fruits except S1 and S2, the peel was separated from the rest of the fruit (flesh) and samples prepared for analysis.

on average), β -carotene (18.9%), and violaxanthin (11.2%). β -Cryptoxanthin was barely detectable in either cultivar.

At the mature green stage (S3), the colour of the peel was green while the flesh was ivory white (Fig. 2) in both cultivars. The total carotenoid levels of LYQ peel increased

and the carotenoid profiles were similar to those at the earlier two stages (S1–S2) except that the content of violaxanthin and 9-cis-violaxanthin decreased. From the breaker (S4) to the fully ripe stage (S6), the total carotenoid content in the peel of LYQ rapidly increased principally due to the

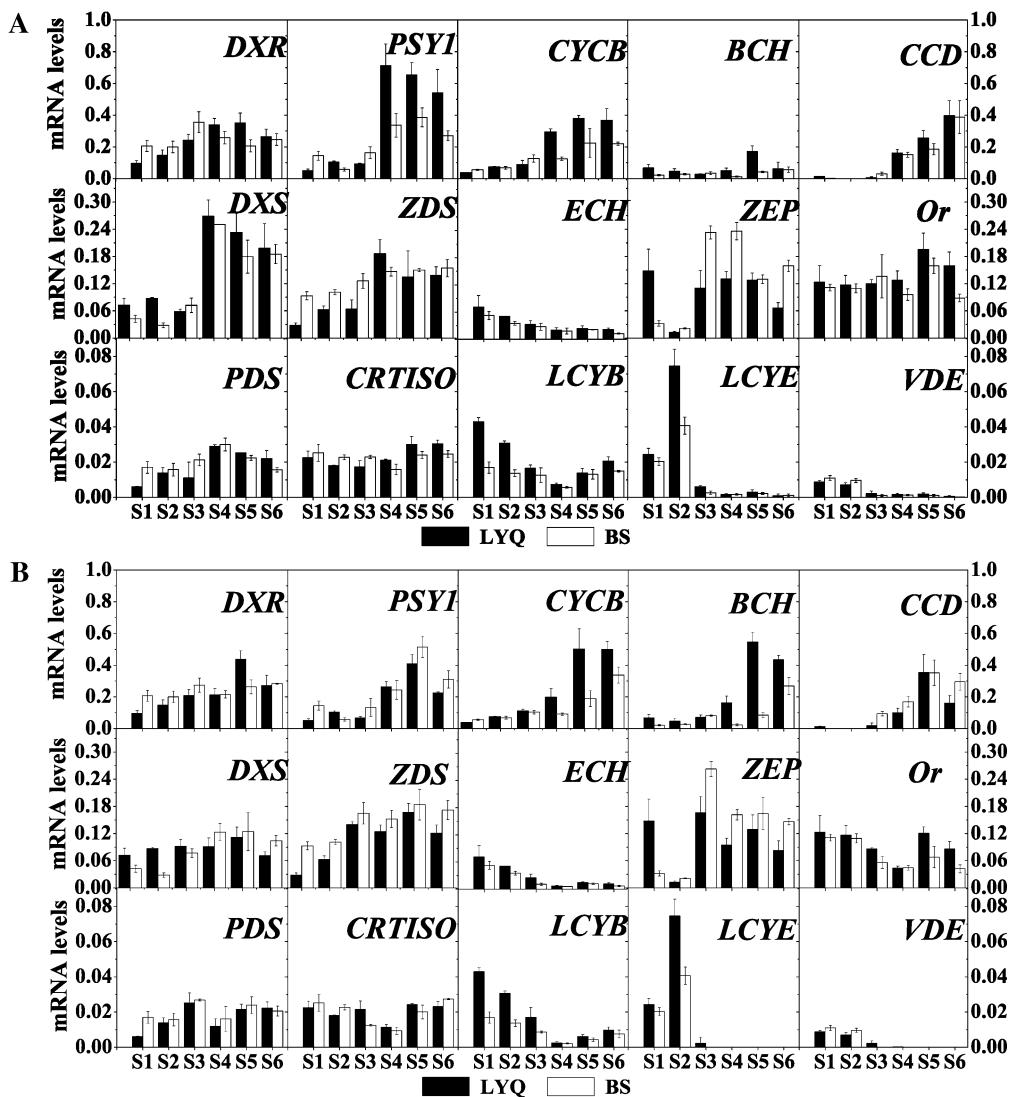


Fig. 4. Expression patterns of carotenoid biosynthetic genes and *Or* gene in LYQ and BS in peel (A) and flesh (B) during fruit development and ripening. The expression level of *actin* was used to normalize the mRNA levels for each sample, with mRNA levels produced by Q-PCR expressed relative to *actin* level. Three replicates were performed for each sample. The fruit ripening stages shown are identical to those depicted in Figs 2 and 3.

increased β -carotene levels during ripening. The content of β -cryptoxanthin, violaxanthin, 9-*cis*-violaxanthin, and lutein also steadily increased. By contrast, the total carotenoid levels in peel of BS showed only a moderate increase mainly due to β -carotene accumulation during ripening (Fig. 3B).

In the flesh of loquat, the total carotenoid content was usually lower than in the peel (Fig. 3A, B). At the S3 stage, the flesh of LYQ and BS contained low total carotenoid levels: $0.94 \mu\text{g g}^{-1}$ fresh weight (FW) for LYQ, and $0.29 \mu\text{g g}^{-1}$ FW for BS, even lower than at the S2 stage. After stage S3, the total carotenoid content in the flesh of LYQ steadily increased from $0.94 \mu\text{g g}^{-1}$ FW to $13.24 \mu\text{g g}^{-1}$ FW, principally due to the increased β -carotene and β -cryptoxanthin levels and, to a lesser extent, violaxanthin and 9-*cis*-violaxanthin, during ripening. However, by contrast with LYQ, the total carotenoid levels in the flesh of BS decreased by 7.1% from stage S3 to S6 (Fig. 3B).

Analysis of carotenogenic gene mRNA abundance in peel and flesh of LYQ and BS fruits

The possibility that differences in carotenoid accumulation in LYQ and BS fruits could be related to the expression of carotenoid-related genes was investigated. Fifteen carotenogenesis-related genes were isolated by traditional PCR and were highly homologous to BLAST matches against *Prunus mume*, and *Malus* species, for which many sequences are available (see Supplementary Tables S1 and S2 at JXB online). The mRNA levels of two genes of the MEP pathway (DXS and DXR) and 12 genes of the carotenoid pathway, as well as the *Or* gene, were analysed in the peel and flesh of LYQ and BS at six developmental and ripening stages (Fig. 4).

Genes (*DXS*, *DXR*) of the MEP pathway showed similar expression patterns in LYQ and BS tissues. The expression levels of *DXR* increased with fruit development in the peel

of LYQ and BS, but peaked at slightly different stages (S3 for BS and S4 for LYQ) (Fig. 4A). In the peel, mRNA levels of *DXS* showed a major increase at the breaker stage (S4) and remained high thereafter, and were slightly higher in LYQ than in BS (Fig. 4A). The expression of the *DXS* gene remained constant in flesh of LYQ, increased slightly in flesh of BS during ripening, and *DXR* expression in the flesh was similar to the peel in both genotypes (Fig. 4B).

In general, genes of the early steps of the pathway (*PDS*, *ZDS*, and *CRTISO*), *ECH*, and *VDE* showed a similar expression profile in LYQ and BS fruit tissues, while differences were detected for *PSYI*, *CYCB*, *LCYB*, *LCYE*, *BCH*, and *ZEP* genes (Fig. 4). The expression of *PSYI* and *CYCB* increased during ripening in the peel of both cultivars, but to a lower level (approximately half) in BS. By contrast, expression of genes of *LCYB* and *LCYE* decreased during ripening in the peel of both cultivars but again to lower levels (approximately half) in BS. In the peel of LYQ, the *BCH* gene mRNA level reached a maximum at stage S5, but in BS the expression of *BCH* was lower and remained constant during fruit ripening. At the S1 stage, the expression level of *ZEP* was lower in the peel of BS than in LYQ. However, the *ZEP* gene expression level was approximately twice as great in the peel of BS as in LYQ thereafter (Fig. 4A). In the flesh, transcripts of *PSYI*, *CYCB*, and *BCH* increased during fruit ripening in both cultivars. However, the *PSYI* gene showed a similar expression profile in flesh of LYQ and BS. The expression of *CYCB* and *BCH* dramatically increased in the flesh of LYQ, but in BS the accumulation of both genes' transcripts was delayed and much reduced (Fig. 4B). In addition, the transcript of *BCH* was more abundant in flesh than in peel (Fig. 4A, B). The expression patterns of *LCYB*, *LCYE*, and *ZEP* were similar in peels and flesh of both cultivars, but the expression of *LCYE* was almost undetectable at the S5 and S6 stages.

The mRNA level of a carotenoid cleavage dioxygenase (*CCD*) gene family member was also analysed. In *Arabidopsis* (Tan *et al.*, 2003), four classes of *CCD* homologues have been reported but, in our study, only one *CCD* member was obtained using degenerate primers for the *CCD* gene and cDNA from mature ripe (S6) LYQ fruit as a template (see Supplementary Table S1 at *JXB* online). mRNA levels of this *CCD* were very low during early fruit development but increased during fruit ripening. The pattern was similar in the peel of the two cultivars, but *CCD* expression was higher in the flesh of BS compared with LYQ during ripening.

The *Or* gene has been reported to control chromoplast differentiation (Lu *et al.*, 2006). A homologous *Or* was isolated using cDNA from flesh of LYQ as template. The expression level of *Or* was found to be slightly higher in LYQ than in BS after the green stages, and was higher in peel than in flesh (Fig. 4).

Light microscopy in LYQ and BS

The reduced carotenogenic gene expression seemed insufficient to explain the big difference in carotenoid content between the

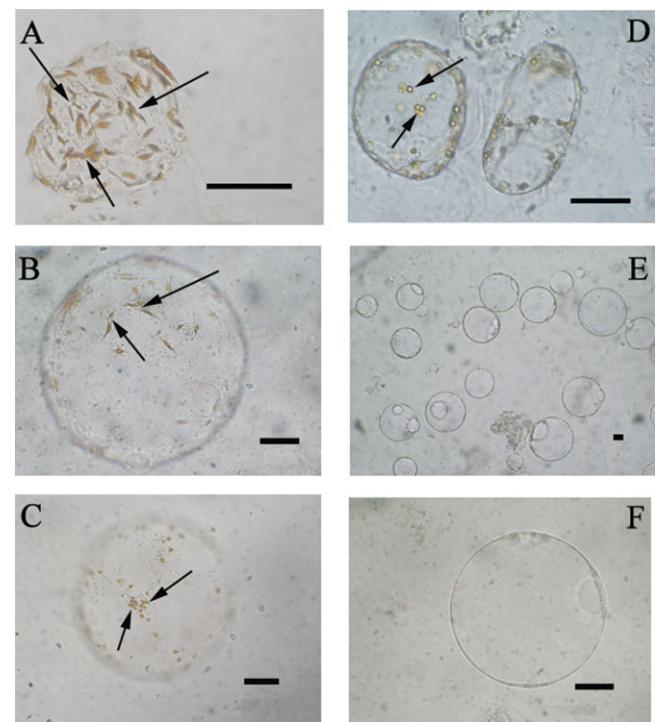


Fig. 5. Light microscopy comparison of chromoplasts between LYQ and BS. Protoplasts were not stained to show the natural colour of chromoplasts (arrows). (A) Protoplasts of peel of LYQ; (B, C) protoplasts of flesh of LYQ; (D) protoplasts of BS peel; (E, F) no chromoplasts were found in the protoplasts of BS flesh. Bar, 20 µm.

two cultivars (e.g. the transcripts of *CYCB* in BS were about half those of LYQ, whereas BS contained 3-fold and 50-fold less carotenoid content in peel and flesh, respectively, compared with LYQ). To examine whether plastid type or quantity is involved in the formation of different carotenoid profiles in LYQ and BS, the plastids in the mature ripe (S6) fruits were examined under the light microscope. About 20–30 orange-red coloured chromoplasts were observed per peel protoplast in LYQ (Fig. 5A), and the chromoplasts were either spindle-like or spherical (see Supplementary Fig. S1A at *JXB* online). Interestingly, individual protoplasts contained plastids of either shape, but not both. By contrast, chromoplasts in the peel of BS under the light microscope appeared yellow in colour, and were present in lower numbers (15–20) and were of smaller size than in LYQ (Fig. 5D). Only spherical chromoplasts were found in the peel of BS (see Supplementary Fig. S1C at *JXB* online). As in the peel, two types of chromoplasts were also found in different cells of the flesh of LYQ (Fig. 5B, C; see Supplementary Fig. S1B at *JXB* online). However, no chromoplasts could be seen in the flesh cells of BS (Fig. 5H, I; see Supplementary Fig. S1D at *JXB* online).

Comparative ultrastructure of plastids in LYQ and BS

To gain further insights into the nature of the plastids in the LYQ and BS fruit tissues, transmission electron microscopy (TEM) was used to observe the ultrastructure of the peel and

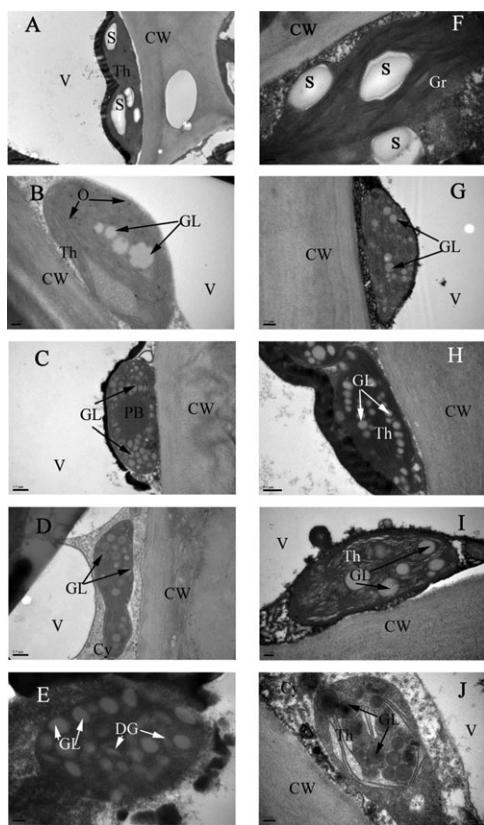


Fig. 6. Transmission electron micrograph of the plastids of peel of loquat fruits at five stages (S2–S6). Cy, cytoplasm; CW, cell wall; DG, developing globules; GL, globules; Gr, grana; O, osmiophilic globules; PB, prolamellar-like body; S, starch grain; Th, thylakoid; V, vacuole. (A) LYQ peel at the immature green stage (S2), bar, 1 µm; (B) LYQ peel at the mature green stage (S3), bar, 0.2 µm; (C) LYQ peel at the breaker stage (S4), bar, 0.5 µm; (D) LYQ peel at the ripe stage (S5), bar, 0.5 µm; (E) LYQ peel at the fully ripe stage (S6), bar, 0.2 µm; (F) BS peel at the immature green stage (S2), bar, 0.2 µm; (G) BS peel at the mature green stage (S3), bar, 0.2 µm; (H) BS peel at the breaker stage (S4), bar, 0.5 µm; (I) BS peel at the half-ripe stage (S5), bar, 0.2 µm; (J) BS peel at the fully ripe stage (S6), bar, 0.2 µm.

flesh cells of both cultivars. As the loquat fruits approached the immature green stage (S2), the plastids showed typical chloroplast morphology in the peel of LYQ and BS, and contained starch grains and intergranal thylakoids and granal thylakoid elements (Fig. 6A, F).

Plastid development in the peel of LYQ and BS

At the S3 stage, chloroplasts began to develop into chromoplasts in the peel cells. In plastids, the starch grains disappeared, globules started to form, although grana arrangements remained (Fig. 6B). As ripening progressed, numerous globules were present in the plastids accompanied by degradation of internal components, and a change in shape of the plastids (Fig. 6C, D). Occasionally, a structure resembling a prolamellar body was seen in the chromoplasts of peel at the breaker stage (S4) in LYQ (Fig. 6C). At the

fully ripe stage (S6), chromoplasts contained a considerably increased number of globules and lost all organization and internal membrane structure. In addition, elongated forms of globules were also found in the chromoplasts (Fig. 6E).

The early development of chromoplasts from chloroplasts in the peel of BS closely paralleled that observed in the peel of LYQ. As ripening progressed, globules were larger in size (average diameter 250 nm at the immature green stage S2 versus 300 nm at the fully ripe stage S6) and increased in number (Fig. 6G, H, I, J). By contrast with LYQ, the chromoplasts in BS still retained internal membranes at the mature ripe stage (S6) (Fig. 6J).

Comparison of plastids at immature green stage (S2) and fully ripe stage (S6) in the flesh of LYQ and BS

At the immature green stage (S2), the plastids in the flesh of LYQ and BS showed abnormal chloroplast morphology with a few distorted internal membranes in the stroma (Fig. 7A, D), and no chloroplast was observed by light microscopy. At the fully ripe stage (S6), chromoplasts in the flesh of LYQ were spindle-shaped and contained empty crystallloid structures. These may have arisen by the removal of crystalline carotene during fixation (Fig. 7B, C). Tubules were also found in the chromoplasts of LYQ flesh. However, unlike the situation in the peel, no globules were found in the chromoplast of LYQ flesh. Occasionally, a membrane-bound, intraplastid body (IB) was observed in the chromoplast of LYQ flesh (Fig. 7C) and also in the smaller plastids of BS (Fig. 7F). By contrast, plastids in the flesh of BS were small, like proplastids, and lacked the crystal-shaped void areas found in the cells of LYQ flesh (Fig. 7E, F), which was consistent with a lack of carotene in the flesh of BS at the maturation stage (S6).

Analysis of PAP mRNA abundance in LYQ and BS cultivars

The plastid lipid-associated protein (related to fibrillin from pepper) gene has been reported to be involved in carotenoid sequestration in plastids (Egea *et al.*, 2010). A homologous *PAP* was isolated using cDNA from LYQ flesh as the template. The mRNA level of *PAP* was found to be distinctly higher (over five times) in LYQ than in BS at the fully ripe stage (Fig. 8).

Discussion

The transcriptional level of carotenogenic genes versus carotenoid accumulation patterns

At the fruitlet and immature green stages (S1 and S2), chloroplast-type carotenoids (mainly lutein, β-carotene, and violaxanthin) accumulated in both LYQ and BS loquat fruits, with high expression levels of *LCYE*, *LCYB*, and *ECH*. Two genes encoding lycopene β-cyclase were obtained in this study. Sequence and phylogenetic analysis indicated that one was the chloroplast-specific lycopene

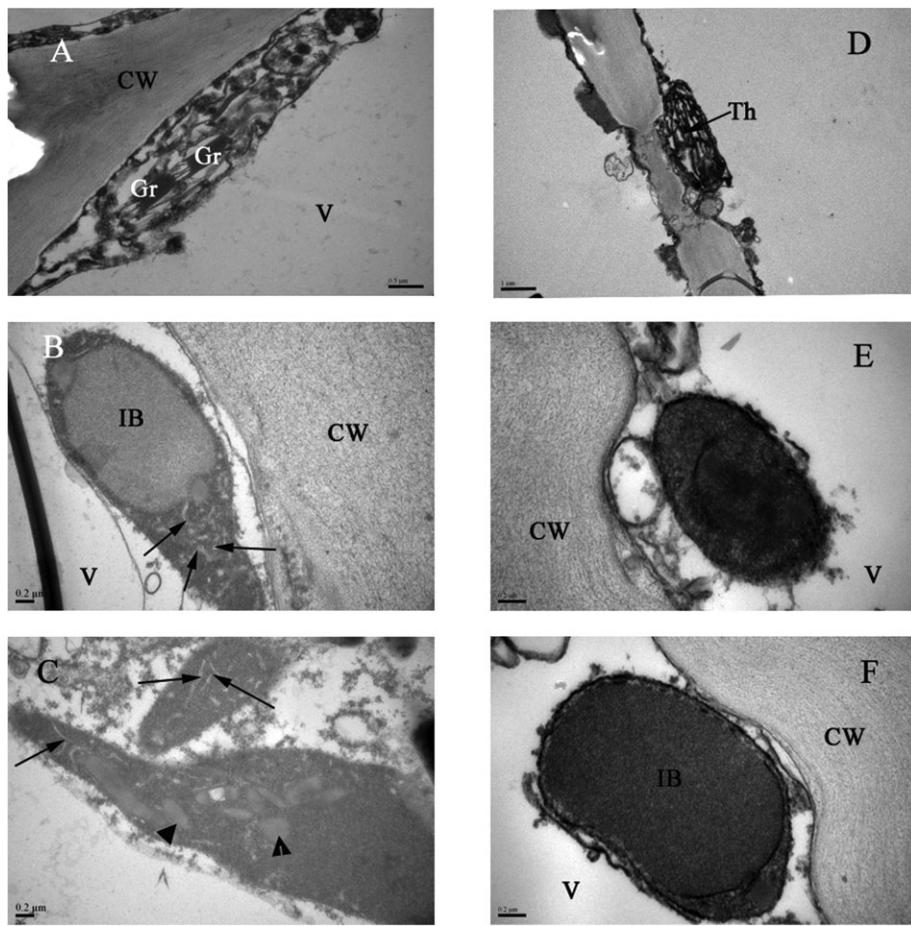


Fig. 7. Transmission electron micrographs of plastids of flesh of LYQ (A–C) and BS (D–F) cultivars at two stages: immature green stage (S2) and fully ripe stage (S6). Arrows, crystal remnants; arrowheads, tubular elements; CW, cell wall; IB, intraplastid body; Th, thylakoid; V, vacuole. (A) Plastids of LYQ flesh at S2, bar, 0.5 μ m; (B, C) plastids of LYQ flesh at S6, bar, 0.2 μ m; (D) plastids of BS flesh at S2, bar, 1 μ m; (E, F) plastids of BS flesh at S6, bars, 0.5 μ m and 0.2 μ m, respectively.

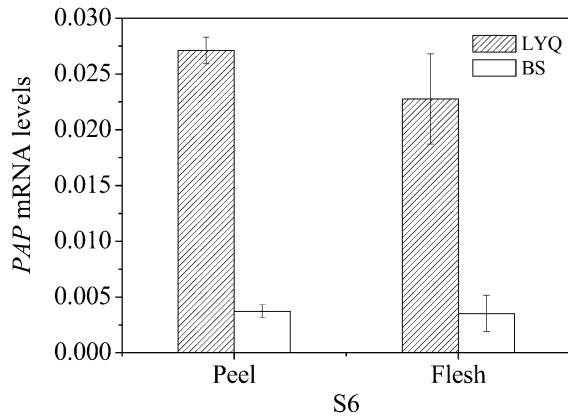


Fig. 8. The plastid lipid-associated protein (PAP) mRNA levels in LYQ and BS peel and flesh at the fully ripe stage (S6).

cyclase named LCYB, and the other was a chromoplast-specific lycopene cyclase designated CYCB (Alquezar *et al.*, 2009). There was little difference between cultivars at this early stage, although the total carotenoid levels in LYQ

fruit were slightly higher than in BS fruit, which corresponded to the higher levels of transcripts of *LCYE*, *LCYB*, and *ECH* in LYQ (Figs 3B, 4).

At the breaker stage (S4), a decrease in lutein with a decline in total carotenoid occurred in peel of both cultivars, which was consistent with the decreased expression levels of *LCYE*, *LCYB*, and *ECH*. After the breaker stage, chromoplast-dependent carotenoids, mainly comprising β , β -carotenoids such as β -carotene, β -cryptoxanthin, + and violaxanthin, started to accumulate in the peel of both cultivars, which might have resulted from the increased gene expression levels of *DXS*, *DXR*, *PSY1*, *ZDS*, *PDS*, and *CYCB* (Figs 3B, 4A). Such variation has been reported previously in mandarin and orange (Kato *et al.*, 2004), where the switch from β , ϵ -carotenoids (α -carotene and lutein) to β , β -carotenoids (β -carotene, β -cryptoxanthin, zeaxanthin, and violaxanthin) accumulation was related to the disappearance of *LCYE* transcripts and the increased expression of genes (*PSY*, *PDS*, *ZDS*, *LCYB*, *BCH*, and *ZEP*) (Kato *et al.*, 2004).

In the peel of LYQ, the total carotenoid levels sharply increased during maturation and ripening mainly due to the

increased β -carotene level. However, the peel of BS only showed a small increase in the total carotenoid level. The higher gene expression levels of *PSY1* and *CYCB* in the peel of LYQ compared with BS, from the breaker stage (S4), might partially explain the higher level of total carotenoid in the peel of LYQ. In a previous report, phytoene synthesis has been considered to be the key step limiting the carotenoid pathway in white carrot (Santos *et al.*, 2005). Similarly, the *PSY* mRNA level was observed to increase 20-fold during the development of tomato fruit (Giuliano *et al.*, 1993). There are two *PSY* genes in tomato; *PSY1* is responsible for the late stages of fruit ripening, while *PSY2* is mainly expressed in green tissues such as mature leaves (Bartley and Scolnik, 1993; Fray and Grierson, 1993). In another study, by deep sequencing (data not shown), two different *PSY* gene sequences with different expression levels were also obtained in loquat. The sequence of one member, identical to *PSY1* cloned by traditional PCR, was highly expressed in mature fruit. The other member, *PSY2*, however, showed a very low level of transcripts in both red and white mature fruits, suggesting it corresponded to the green tissues phytoene synthase. *CYCB* is also important in β -carotene production, and the level of *CYCB* transcripts was higher in LYQ than in BS. In the *Beta* (B) tomato mutant, the dramatically increased expression of *CYCB* at the breaker stage caused high levels of β -carotene to accumulate instead of lycopene (Ronen *et al.*, 2000), and this may also happen to some extent in the LYQ loquat.

Total carotenoids in the flesh of both cultivars showed lower levels compared with those in the peel during development, although the gene expression profile in the flesh was similar to that of the peel. This differs from the results of Alquezar *et al.* (2008), who found that all genes investigated in Cara Cara and Navel orange fruits showed higher transcripts in flavedo than in pulp during fruit development. This suggests that, in loquat, other factors, in addition to gene expression levels, govern carotenoid accumulation. From the breaker stage (S4) to the fully ripe stage (S6), total carotenoid content gradually increased in the flesh of LYQ. In the flesh of BS, by contrast, the total carotenoid levels did not increase and remained low during ripening. The expression of carotenogenic genes in the flesh of LYQ and BS was followed during fruit development. The most prominent feature was the difference in *BCH* and *CYCB* expression between the flesh of LYQ and BS. The transcripts of *BCH* drastically increased at the breaker stage (S4) in the flesh of LYQ while it remained low and showed a delayed increase in the flesh of BS. Meanwhile, the expression level of *CYCB* was also higher in the flesh of LYQ than in BS, after the breaker stage (S4). According to previous studies, the *BCH* gene plays a major role in carotenoid accumulation in various plants. Low transcription of *BCH* in morning glory (*Ipomoea nil*) resulted in white coloured flowers which lack the ability to synthesize chromoplast-type carotenoids (Yamamoto *et al.*, 2010). The tomato mutant *white-flower* (*wf*) showed an 80% reduction in total carotenoid concentration, caused by a mutation in *CrtR-b2*, a *BCH* homologue that is specifically expressed in

petals. However, expression of other genes participating in the carotenoid biosynthetic pathway was not altered (Galpaz *et al.*, 2006).

In chrysanthemums, *CmCCD4a* contributes to white colour formation in petals by cleaving carotenoids into colourless compounds (Ohmiya *et al.*, 2006). Similarly, higher expression of *CCD4* is responsible for the much reduced accumulation of carotenoids in a white-fleshed peach mutant (Brandi *et al.*, 2011). In our study, however, similar expression profiles of *CCD* were detected in the peel of both cultivars during ripening. Although transcripts of *CCD* accumulated to a slightly higher level in the flesh of BS than in LYQ, this was not sufficient to cause the low pigmentation in flesh of BS.

The *or* mutation in cauliflower (*Brassica oleracea* var. *botrytis*) leads to the abnormal accumulation of β -carotene in tissues which are unpigmented in wild-type plants (Li *et al.*, 2001). The *Or* gene product has been reported as a molecular switch to trigger chromoplast differentiation (Giuliano and Diretto, 2007), and for this reason the *Or* expression level was examined in the present study. mRNA levels of *Or* showed no significant differences between LYQ and BS at the green stage, whereas, they were slightly higher in LYQ compared with BS tissues after the breaker stage as the chromoplasts started to form. The *Or* gene, therefore, might have some contribution to additional carotenoid accumulation in LYQ loquat, but the presence of transcripts in the flesh of BS suggests either that it is non-functional in this fruit or there must be a different explanation for the failure to form chromoplasts.

The expression levels of *CYCB* or *BCH* genes in LYQ were about 2-fold higher than in BS. This is not sufficient to explain the almost 50-fold higher carotenoid content in LYQ flesh compared with BS, and a different explanation is required, such as an altered developmental event involved in the biogenesis of the chromoplast.

Plastids ultrastructure

In higher plants, the complete pathway of carotenoid biosynthesis takes place within the plastid, where carotenoids are localized and carotenoid accumulation depends on the level of gene expression and enzyme activity and also on the size and number of plastids (Kim *et al.*, 2010). Kim *et al.* (2010) found that white carrot roots contained significantly fewer chromoplasts than the orange varieties. However, numerous starch-filled amyloplasts were found in the white variety. Schweiggert *et al.* (2011) also discovered different chromoplast morphology and ultrastructure between red- and yellow-fleshed papaya. Previous studies have shown that the increases in carotenoid accumulation were associated more with the proliferation of carotenoid sequestering structures than with changed carotenogenic gene expression or enzyme abundance (Rabbani *et al.*, 1998; Li *et al.*, 2001). In the present study, plastids in LYQ and BS were compared using light and transmission electron microscopy (TEM). Light microscopy (Fig. 5) indicated that mature chromoplasts in the peel of LYQ were orange in colour which suggested a high

level of carotenoids, while mature chromoplasts in the peel of BS appeared yellow which suggested a low level of carotenoid (Fig. 3A). Mature chromoplasts in the flesh of LYQ were similar in appearance to those in the peel: orange in colour with two morphological, distinct types of chromoplast, one spindle-like and the other spherical. The carotenoid composition was probably different between the two types but it will be necessary to develop methods to isolate them before this can be tested. In addition, it was noted that one cell contained only one chromoplast type, suggesting perhaps that the shape or content was controlled by a gene encoded in the nucleus. No chromoplast was found in the flesh of BS, which was consistent with the low carotenoid accumulation (Fig. 3A), but it is not possible to discount the presence of small undeveloped plastids.

Differences in ultrastructure between LYQ and BS peel chromoplasts were also observed. Mature chromoplasts of the LYQ peel usually had few membranes but many globules while, in mature chromoplasts of the BS peel, the membrane system still remained and only a moderate number of globules were observed (Fig. 6). These globules, which increased in number during ripening, were the main sites of pigment deposition. Corresponding findings have also been published for other plants, for example, large and numerous globules are found in a β -carotene-rich tomato mutant, which supports the view of a globule location for pigment accumulation (Harris and Spurr, 1969). Interestingly, *PAP* mRNA level in LYQ peel was over five times higher than the peel of BS. Simkin *et al.* (2007) found that over-expression in tomato of a pepper plastoglobulin, fibrillin, resulted in an increase in carotenoid and carotenoid-derived flavour volatiles. There is other evidence that the suppression of CHRC results in a 30% reduction of carotenoids in tomato flowers compared with the wild type (Leitner-Dagan *et al.*, 2006).

Thus, plastid ultrastructure in the peel of BS showed less change than in LYQ during development and ripening and the lower expression of the *PAP* gene in BS was better correlated with the low carotenoid content in BS rather than reduced carotenogenic gene expression. Interestingly, in the flesh of LYQ, carotenoids were shown to accumulate in crystalline and tubule forms in the chromoplasts (Fig. 7) while only the globular chromoplast type was found in the loquat peel. This suggests the different carotenoid composition patterns between the peel and flesh might be related to the differences in chromoplast ultrastructure. It has been reported that chromoplasts of tomato flowers and fruits differ in both the structure of the internal membranes and the carotenoid composition. Different chromoplast structures were also found in different carotenoid composition of the red- and yellow-fleshed papaya (Schweiggert *et al.*, 2011). Beta-carotene was shown to accumulate in the crystalline form and spherical lipoidal globules were also present in the chromoplasts of cultured carrot cells (Israel *et al.*, 1969) and, in carrot roots, carotene accumulates in the crystalline form in chromoplasts (Kim *et al.*, 2010). In fruits of pepper (*Capsicum annuum*) β -carotene is concentrated within glob-

ules (Simpson *et al.*, 1974, 1978a, b). There were some similarities between the developing chromoplasts in the flesh of LYQ and leucoplasts, suggesting that leucoplasts or proplastids developed into chromoplasts in these tissues, whereas in BS flesh there were very few leucoplasts or proplastids and there were no chromoplasts. This is consistent with the suggestion that, in papaya (Schweiggert *et al.*, 2011), the chromoplasts are likely to emerge directly from proplastids. At the fully ripe stage, the *PAP* expression in the flesh of BS was over five times less than in LYQ flesh, indicating that there were fewer chromoplasts in the flesh of BS. Exactly how *PAP* is involved in the formation of chromoplasts during loquat development requires further study. It is clear, however, that the inability to form chromoplasts in the flesh of BS is the mostly likely explanation for the low carotenoid accumulation in BS compared with LYQ flesh.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. PCR amplification of carotenogenesis related genes.

Supplementary Table S2. Homologies based on nucleotide sequences for carotenoid biosynthesis genes and the *PAP* gene isolated from LYQ loquat fruit.

Supplementary Table S3. The CIE colour values of loquat peels for LYQ and BS.

Supplementary Fig. S1. Chromoplast extracts from loquat fruit tissues.

Acknowledgements

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