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ORIGINAL ARTICLE

Expression and functional analysis of two lycopene β-cyclases from citrus fruits

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Abstract In the present study, two LCYb genes (*CitLCYb1* and *CitLCYb2*) were isolated from Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*Citrus sinensis* Osbeck) and Lisbon lemon (*Citrus limon* Burm.f.) and their functions were analyzed by the color complementation assay in lycopene-accumulating *E. coli* cells. The results showed that *CitLCYb1* and *CitLCYb2* shared high identity at the amino acid level among the three citrus varieties. The N-terminal region of the two proteins encoded by *CitLCYb1* and *CitLCYb2* was predicted to contain a 51-residue chloroplastic transit peptide, which shared low similarity. In Satsuma mandarin, the secondary structures of the *CitLCYb1* and *CitLCYb2* encoding proteins without the transit peptide were quite similar. Moreover, functional analysis showed that both enzymes of *CitLCYb1* and *CitLCYb2* participated in the formation of β-carotene, and when they were co-expressed with *CitLCYe*, α-carotene could be produced from lycopene in *E. coli* cells. However, although *CitLCYb2* could convert lycopene to α-carotene in *E. coli* cells, its extremely low level of expression indicated that *CitLCYb2* did not participate in the formation of α-carotene during the green stage in the flavedo. In addition, the high expression levels of *CitLCYb1* and *CitLCYb2* during the orange stage played an important role in the accumulation of β,β-xanthophylls in citrus fruits. The results presented in this study might contribute to elucidate the mechanism of carotenoid accumulation in citrus fruits.

Keywords Carotenoid · Citrus · Lycopene β-cyclase · α-Carotene · β-Carotene

Abbreviations

ABA	Abscisic acid
GGPP	Geranylgeranyl diphosphate
HYb	β-Ring hydroxylase
HYe	ε-Ring hydroxylase
LCY	Lycopene cyclase
LCYb	Lycopene β-cyclase
LCYe	Lycopene ε-cyclase
PDS	Phytoene desaturase
PSY	Phytoene synthase
ZDS	ζ-Carotene desaturase
ZEP	Zeaxanthin epoxidase
α-Car	α-Carotene
β-Car	β-Carotene

Introduction

Carotenoids are important natural isoprenoid pigments, which provide distinct yellow, red and orange colors to

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flowers and fruits (Ronen et al. 2000; Schweiggert et al. 2011). In addition, carotenoids fulfill a variety of other critical functions in plants, such as the stabilization of lipid membranes, light harvesting for photosynthesis, as well as protecting the photosystem from photo-oxidation (Havaux 1998; Harvaux and Kloppstech 2001; Ledford and Niyogi 2005). Carotenoids are also the precursors of the plant hormone abscisic acid (ABA) (Schwartz et al. 1997; Cunningham and Gantt 1998). Carotenoids are not only important to the plants themselves, but also beneficial to human health. Some carotenoids with a β-ring are the precursors of vitamin A, which is a fundamental nutrient for humans (Giovannucci 1999; Krinsky et al. 2003). Recent studies have identified that the benefits from carotenoids might be due to β-cryptoxanthin, which is one of the major carotenoids in human blood (Fu et al. 2010). Altucci and Gronemeyer (2001) reported that β-cryptoxanthin played an important role in the prevention of some diseases, especially cancers, because of its antioxidant activity. Additionally, β-cryptoxanthin served as a retinoic acid receptor (RAR) ligand and exerted beneficial effects on atherogenesis through RAR activation (Matsumoto et al. 2007).

The pathway of carotenoid biosynthesis is a series of desaturation, cyclization, hydroxylation, and epoxidation steps (Cunningham and Gantt 1998; Kato et al. 2004). Genes encoding the enzymes in the pathway have been cloned and their expression profiles have also been well characterized in citrus fruits (Kato et al. 2004, 2006; Fig. 1). The cyclization of lycopene is a key branch point in the carotenoid biosynthetic pathway (Fig. 1). In citrus fruits, a massive amount of β,ε-carotenoid is accumulated in the flavedo during the green stage. With the transition from the green stage to the orange stage, the pathway shifts from β,ε-carotenoid synthesis to β,β-carotenoid synthesis, resulting in the accumulation of β,β-xanthophylls in citrus fruits (Kato et al. 2004; Inoue et al. 2006). Two enzymes, lycopene ε-cyclase (LCYe) and lycopene β-cyclase (LCYb), which share high similarity in amino acid sequence and likely evolved from the same ancestor, have been confirmed to catalyze the cyclization of lycopene (Sandmann 2002). LCYe adds one ε-ring to form the monocyclic δ-carotene, which is a substrate for LCYb to form α-carotene (Fig. 1). LCYb can also add two β-rings to lycopene, leading to the biosynthesis of β,β-carotenoids (Fig. 1). In pepper, capsanthin–capsorubin synthase (CCS), which shares high identity at the amino level with LCYb, also has LCYb activity and is highly induced during fruit coloration (Hugueney et al. 1995; Mialoundama, et al. 2010; Mendes, et al. 2011). Recently, two LCYb genes (*LCYb1* and *LCYb2*) have been isolated in some plants, such as tomato, papaya and citrus (Pecker et al. 1996; Ronen et al. 2000; Tadmor et al. 2005; Alquézar et al. 2009; Devitt et al. 2010; Mendes et al. 2011). It was reported that the enzymes encoded by *LCYb1* and *LCYb2* could generate β-carotene from lycopene in *E. coli* cells (Ronen et al. 2000; Alquézar et al. 2009; Devitt et al. 2010). To date, however, the roles of *LCYb1* and *LCYb2* in the production of α-carotene are still unclear.

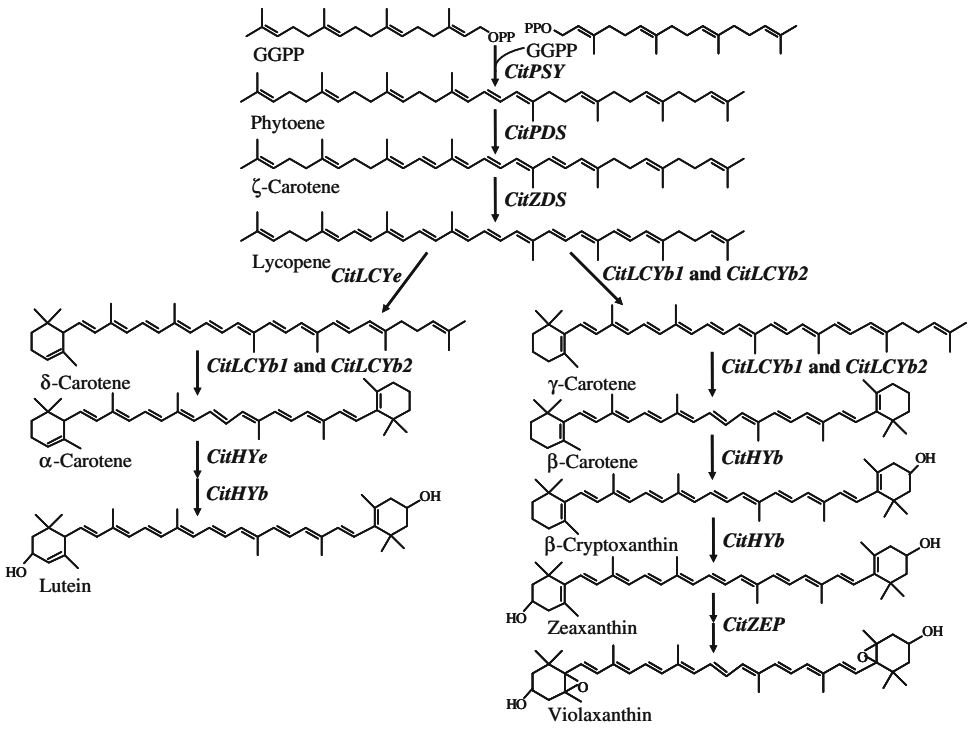

Fig. 1 Carotenoid metabolic pathway in citrus. *GGPP* geranylgeranyl diphosphate, *PSY* phytoene synthase, *PDS* phytoene desaturase, *ZDS* ζ-carotene desaturase, *LCYb* lycopene β-cyclase, *LCYe* lycopene ε-cyclase, *HYe* ε-ring hydroxylase, *HYb* β-ring hydroxylase, *ZEP* zea epoxidase

Citrus is one of the richest sources of carotenoids in plants. The content and composition of carotenoids, which are important indexes for the commercial and nutritional quality of citrus fruits, vary greatly among different citrus species and varieties. In Satsuma mandarin (*Citrus unshiu* Marc.), β-cryptoxanthin is accumulated predominantly in juice sacs, while in Valencia orange (*Citrus sinensis* Osbeck), violaxanthin isomers are the principal carotenoids (Molnár and Szabolcs 1980; Goodner et al. 2001; Lee and Castle 2001; Kato et al. 2004). In Lisbon lemon (*Citrus limon* Burm.f.), the carotenoid content is much lower than that in Satsuma mandarin or Valencia orange. These citrus varieties are useful for investigating the mechanism of carotenoid accumulation because of their different carotenoid profiles (Kato et al. 2004, 2006; Zhang et al. 2012). In the present study, to further elucidate the functions of *LCYb1* and *LCYb2* in carotenoid biosynthesis, we isolated *CitLCYb1* and *CitLCYb2* from three citrus varieties, Satsuma mandarin, Valencia orange and Lisbon lemon. Functional analyses of these two genes from the three citrus varieties were conducted using a color complementation assay in lycopene-accumulating *E. coli* cells. Changes in the expression of *CitLCYb1* and *CitLCYb2* in the flavedo and juice sacs in the three citrus varieties during natural ripening were also examined. The results presented in this study might contribute to elucidate the mechanism of carotenoid accumulation in citrus fruits.

Materials and methods

Plant materials

Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*Citrus sinensis* Osbeck) and Lisbon lemon (*Citrus limon* Burm.f.) cultivated at the National Institute of Fruit Tree Science, Department of Citrus Research, Okitsu (Shizuoka, Japan) were used as materials. Fruit samples were collected periodically from August to January for Satsuma mandarin and from August to February for Valencia orange and Lisbon lemon. The flavedos and juice sacs were separated from sampled fruits, immediately frozen in liquid nitrogen, and kept at −80 °C until used.

Isolation of *CitLCYb1*, *CitLCYb2* and *CitLCYe*

Total RNA was extracted from the flavedos of Satsuma mandarin, Valencia orange and Lisbon lemon fruits according to the method described by Ikoma et al. (1996). First-strand cDNA was synthesized from 2 μg of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The cDNA fragments of *CitLCYb1*, *CitLCYb2* and *CitLCYe* were amplified by PCR using a cDNA template (Kato et al. 2007; Alquézar et al. 2009). The amplified cDNAs were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Sequence analysis of *CitLCYb1* and *CitLCYb2*

The alignments of *CitLCYb1* and *CitLCYb2* among the three citrus varieties were created using the Genetyx Analysis Program (Genetyx Corp., Tokyo, Japan). The information regarding gene structure was obtained from the Citrus Genome Database (http://www.citrusgenomedb.org). Predictions of transit peptides of *CitLCYb1* and *CitLCYb2* were carried out using TargetP. The structures of proteins encoded by *CitLCYb1* and *CitLCYb2* were predicted by SWISS-MODEL server (http://swissmodel.expasy.org) using the loading template 3atr.pdb. The structural analyses were performed using Molegro Molecular Viewer.

Functional analysis of *CitLCYb1* and *CitLCYb2* in *E. coli* cells

The *CitLCYb1* and *CitLCYb2* cDNAs containing the complete coding sequence from Satsuma mandarin, Valencia orange and Lisbon lemon were cloned into pGEX-6p-1 vector, respectively. The full length of *CitLCYe* cDNA from the three citrus varieties was cloned into the recombinant plasmids harboring *CitLCYb1* or *CitLCYb2*. The recombinant plasmids constructed in the present study were designated as: pGEX-6p-1-*CitLCYb1*, pGEX-6p-1-*CitLCYb2*, pGEX-6p-1-*CitLCYb1*+*CitLCYe* and pGEX-6p-1-*CitLCYb2*+*CitLCYe*. The four recombinant plasmids were transformed into lycopene-accumulating *E. coli* XL1-Blue cells harboring a lycopene biosynthetic plasmid pACCRT-EIB (Misawa and Shimada 1997). The *E. coli* XL1-BLUE-EIB cells with pGEX-6p-1 (empty vector) were used as a control. The transformants were plated in LB medium supplemented with chloramphenicol (50 μg ml^{-1}) and carbenicillin (50 μg ml^{-1}), and incubated at 37 °C for 20 h. The colonies were incubated in 100 ml of 2× YT medium (l^{-1} 16 g tryptone, 10 g yeast extract, and 5 g NaCl) with chloramphenicol (50 μg ml^{-1}) and carbenicillin (50 μg ml^{-1}) at 37 °C for 16 h. Then, 2 ml of culture solution was inoculated into 200 ml of 2× YT medium with chloramphenicol (50 μg ml^{-1}) and carbenicillin (50 μg ml^{-1}). After being cultured for 8 h at 27 °C, 200 μl of 0.1 M isopropyl β-D-thiogalactoside was added and the cells were cultured overnight at 27 °C.

Extraction and determination of carotenoids

Cultures of *E. coli* cells were centrifuged at 5,000 *g* for 10 min and the bacterial pellet was washed twice with

Tris–HCl (pH 8.0). The pellet was dried using vacuum freeze drying and stored at −20 °C until HPLC analysis. The freeze-ground material was extracted with a mixture of chloroform and methanol (2:1 by vol.) until all the color was removed from the *E. coli* cells. The carotenoids extracts were reduced to dryness by rotary evaporation, and then dissolved in the methyl tertbutyl ether:methanol (1:1 by vol.) solution containing 0.1 % butylated hydroxytoluene. The identification and quantification of carotenoids were conducted according to the methods described by Kato et al. (2004).

Total RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from the flavedos and juice sacs of Satsuma mandarin, Valencia orange and Lisbon lemon fruits at different stages according to the method described by Ikoma et al. (1996). The total RNA was cleaned up with the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion. The reactions of reverse transcription (RT) were performed with 2 μg of purified RNA and a random hexamer at 37 °C for 60 min using TaqMan Reverse Transcription Reagents (Applied Biosystems).

TaqMan MGB probes and sets of primers for *CitLCYb1*, *CitLCYb2* and *CitLCYe* were designed on the basis of the conserved sequences among the three varieties for each gene with the Primer Express software (Applied Biosystems; Kato et al. 2007; Alquézar et al. 2009). For the endogenous control, the TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems) was used. TaqMan real-time PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using ABI PRISM 7300 (Applied Biosystems) according to the manufacturer's instructions. Each reaction contained 900 nM of the primers, 250 nM of the TaqMan MGB Probe, and template cDNA. The thermal cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The levels of gene expression were analyzed with ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems) and normalized with the results of 18S ribosomal RNA. Real-time quantitative RT-PCR was performed in three replicates for each sample.

Results

Isolation and sequence analysis of *CitLCYb1* and *CitLCYb2*

Two lycopene β-cyclase genes (*CitLCYb1* and *CitLCYb2*) were isolated from three citrus varieties, Satsuma mandarin (accession no. AB114652 and AB719392), Valencia orange (accession no. AB114660 and AB719393) and Lisbon lemon (accession no. AB114668 and AB719394) and their nucleotide and amino acid sequences were analyzed using the Genetyx Analysis Program. As shown in Fig. 2, *CitLCYb1* and *CitLCYb2* showed high homology at the amino acid level among Satsuma mandarin, Valencia orange and Lisbon lemon. The identities of *CitLCYb1* and *CitLCYb2* between Satsuma mandarin and Valencia orange were 98 and 97 %, respectively. The identities of *CitLCYb1* and *CitLCYb2* between Satsuma mandarin and Lisbon lemon were a little lower, which were 96 and 93 %, respectively (Fig. 2). The nucleotide sequence of *CitLCYb1* contained 1,512 bp, and encoded a putative protein of 504 amino acids with an estimated molecular mass of 56 kDa. The nucleotide sequence of *CitLCYb2* contained 1,506 bp, encoding a putative protein of 502 amino acids with a calculated molecular mass of 56 kDa. A BLAST search in Citrus Genome Database (http://www.citrusgenomedb.org) revealed that the sequences of *CitLCYb1* and *CitLCYb2* were identical to scaffold_13:1128474:1130250 and scaffold_73:204264:206149, respectively. In *CitLCYb1*, an intron was detected in the upstream close to the start codon. However, no intron was observed in *CitLCYb2*.

In Satsuma mandarin, *CitLCYb1* and *CitLCYb2* showed 53 % identity at the amino acid level (Fig. 3). The N-terminal region of the two proteins encoded by *CitLCYb1* and *CitLCYb2* was predicted to have a 51-residue chloroplastic transit peptide, which shared very low similarity. Without this peptide, *CitLCYb1* and *CitLCYb2* shared 83 % identity at the amino acid level. The protein structures of the *CitLCYb1* and *CitLCYb2* generated using the SWISS-MODEL protein-modeling server showed that without the transit peptide, the secondary structures of the proteins encoded by *CitLCYb1* and *CitLCYb2* were quite similar. Two β-sheets, which comprised four or five anti-parallel β-stands, were observed in the proteins encoded by *CitLCYb1* and *CitLCYb2*. Six α-helixes of similar length located in the C-terminal region of the two proteins (Supplemental Fig. S1). In addition, a single loop formed by the five anti-parallel β-stands, which might be the active sites, was observed in the proteins encoded by *CitLCYb1* and *CitLCYb2* (Supplemental Fig. S1).

Functional analysis of *CitLCYb1* and *CitLCYb2*

To investigate the functions of *CitLCYb1* and *CitLCYb2*, the cDNAs of *CitLCYb1* and *CitLCYb2* isolated from the three citrus varieties were cloned into pGEX-6p-1 vector. The recombinant plasmids with *CitLCYb1* or *CitLCYb2* were further ligated with the PCR product of *CitLCYe* containing the SD sequence in N-terminal region. The pGEX-6p-1 vector without the insert, and the recombinant

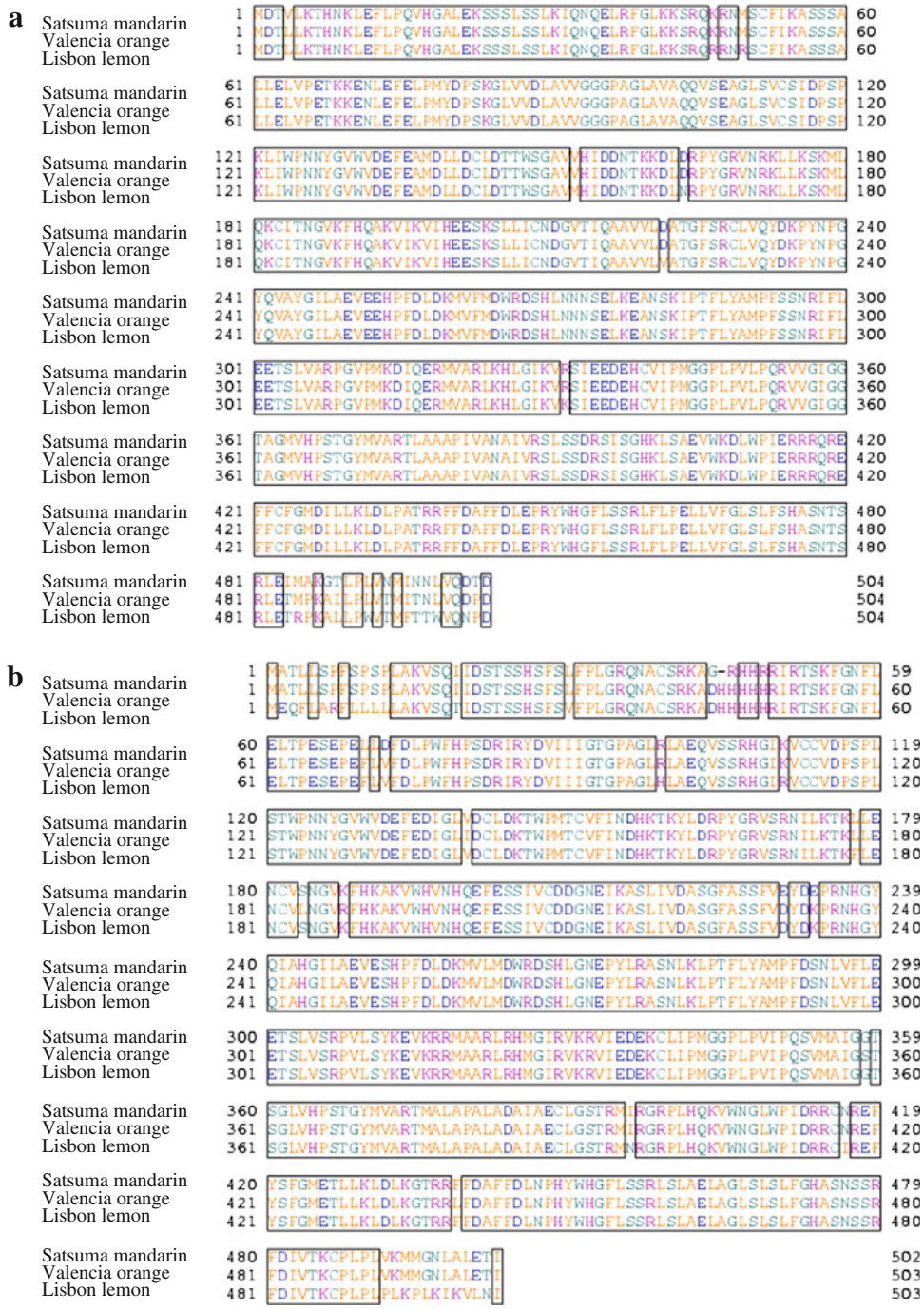

Fig. 2 Alignment of deduced amino acid sequences of *CitLCYb1* (**a**) and *CitLCYb2* (**b**) among Satsuma mandarin, Valencia orange and Lisbon lemon. The alignments of *CitLCYb1* and *CitLCYb2* among the three citrus varieties were created using the Genetyx Analysis Program (Genetyx Corp., Tokyo, Japan)

plasmids (pGEX-6p-1-*CitLCYb1*, pGEX-6p-1-*CitLCYb2*, pGEX-6p-1-*CitLCYb1*+*CitLCYe*, and pGEX-6p-1-*CitLCYb2*+*CitLCYe*) were transformed to the lycopene-accumulating *E. coli* XL1-BLUE-EIB cells. Carotenoids were extracted from bacteria and their contents and compositions were analyzed by HPLC. As shown in Fig. 4a, the peak of lycopene eluted at 85 min was observed in the extract solution of the control. In the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids pGEX-6p-1-*CitLCYb1* from the three citrus varieties, *β*-carotene was detected, while the peak of lycopene disappeared. In the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids pGEX-6p-1-*CitLCYb2* from the three citrus varieties, *β*-carotene was detected (Fig. 4b). Meanwhile, in the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids pGEX-6p-1-*CitLCYb2* from Satsuma mandarin and Valencia orange, a small peak of lycopene was detected. In the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids pGEX-6p-1-*CitLCYb1*+*CitLCYe*

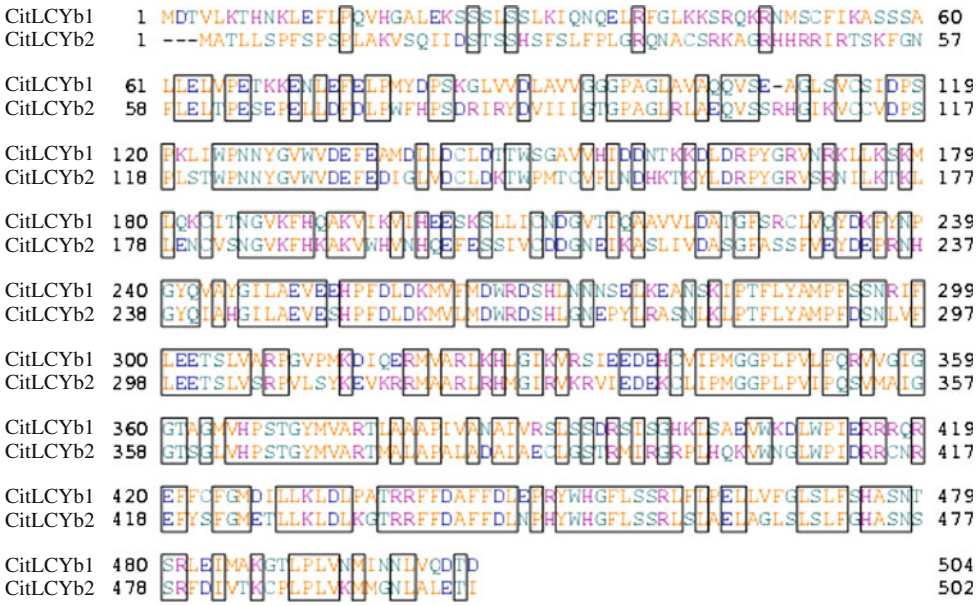

Fig. 3 Alignment of deduced amino acid sequences of *CitLCYb1* and *CitLCYb2* in Satsuma mandarin. The alignments of *CitLCYb1* and *CitLCYb2* were created using the Genetyx Analysis Program (Genetyx Corp., Tokyo, Japan)

from Satsuma mandarin, Valencia orange and Lisbon lemon, peaks of β-carotene and α-carotene were observed (Fig. 5a). The content of β-carotene was higher than that of α-carotene in the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids pGEX-6p-1-*CitLCYb1*+*CitLCYe* from Satsuma mandarin and Lisbon lemon. In the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids pGEX-6p-1-*CitLCYb2*+*CitLCYe* from the three citrus varieties, the peak of α-carotene was observed, which was much higher than that of β-carotene (Fig. 5b).

Gene expression of *CitLCYb1*, *CitLCYb2* and *CitLCYe* in citrus fruits during fruit ripening

According to the changes in the color of the flavedo, the ripening process of the citrus fruits can be divided into two stages, a green stage and an orange stage. The green stages in Satsuma mandarin, Valencia orange and Lisbon lemon were from August to September, from August to October, and from August to October, respectively (Kato et al. 2004, 2006).

In the flavedo, the expression of *CitLCYb1* increased rapidly, while that of *CitLCYb2* remained low level during the green stage in Satsuma mandarin, Valencia orange and Lisbon lemon. The expression of *CitLCYe*, which increased significantly during the green stage, had a similar pattern to that of *CitLCYb1* in the three citrus varieties (Fig. 6a). During the orange stage, the expression of *CitLCYb1* and *CitLCYb2* increased to a maximum, while that of *CitLCYe* decreased significantly to a low level in Satsuma mandarin, Valencia orange and Lisbon lemon (Fig. 6a). In the juice sacs, the gene expression of *CitLCYb1* and *CitLCYb2* increased rapidly in the green stage, and reached a maximum in the orange stage in Satsuma mandarin, Valencia orange and Lisbon lemon (Fig. 6b). In addition, in Satsuma mandarin, the gene expression levels of *CitLCYb1* and *CitLCYb2* were much higher than those in Valencia orange and Lisbon lemon in the flavedo and juice sacs (Fig. 6).

Discussion

Sequence analysis of *CitLCYb1* and *CitLCYb2* from the three citrus varieties

The presence of two LCYb genes has been reported in some plants, such as tomato, papaya and citrus (Pecker et al. 1996; Ronen et al. 2000; Tadmor et al. 2005, Alquézar et al. 2009; Devitt et al. 2010; Mendes et al. 2011). In citrus, the phylogenetic tree showed that the two LCYb genes were clustered into two different subfamilies. LCYb1 was grouped with the plant β-LCYs cluster, while LCYb2 was in the same group with *solanum* NSYs, tomato CYC-B and pepper CCS (Alquézar et al. 2009; Mendes et al. 2011). In the present study, the two LCYb genes (*CitLCYb1* and *CitLCYb2*) were isolated from Satsuma mandarin, Valencia orange and Lisbon lemon and their sequences were analyzed. The results showed that *CitLCYb1* and *CitLCYb2* shared high identity (more than 93 %) at the amino acid level among the three citrus varieties (Fig. 2). The N-terminal region of the proteins encoded by *CitLCYb1* and *CitLCYb2* was predicted to contain a transit peptide, which shared low similarity between the two genes. It has been reported that *LCYb1* and *LCYb2* expressed in different parts of the cell (Alquézar

et al. 2009; Mendes et al. 2011). *LCYb1* expressed in chloroplast, while *LCYb2* expressed in chromoplast. Thus, the low similarity between the transit peptides encoded by *CitLCYb1* and *CitLCYb2* might be due to the different locations of the two genes. In Satsuma mandarin, without the transit peptide, the secondary structures of the *CitLCYb1* and *CitLCYb2* encoding proteins were quite similar (supplemental Fig. S1). A single loop formed by the five anti-parallel β-stands was observed in both proteins encoded by *CitLCYb1* and *CitLCYb2* (Supplemental Fig. S1). Liang et al. (2006) found that this single loop was conserved in LCYb and LCYe in cyanobacteria and plants, which might be related to binding domains. Moreover, some key structural and functional domains, such as conserved plant β-LCY regions, a dinucleotide-binding domain and cyclase motifs, were identified in both LCY1 and LCY2 in citrus fruits (Alquézar et al. 2009; Mendes et al. 2011). Thus, the similar structure and functional domains of LCY1 and LCY2 indicated that the two LCYb genes might have similar functions in the biosynthesis of carotenoids in citrus fruits.

Functional analysis of *CitLCYb1* and *CitLCYb2* in *E. coli* cells

The cyclization of lycopene, a key branch point in the pathway of carotenoid biosynthesis in citrus fruits, involves two lycopene cyclases (LCY), β-cyclase (LCYb) and ε-cyclase (LCYe) (Cunningham et al. 1996; Bai et al. 2009). In the present study, functional analysis showed that in the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with pGEX-6p-1-*CitLCYb1* from the three citrus varieties, only β-carotene was observed, the peak of lycopene having disappeared completely (Fig. 4a). These results suggested that the enzyme encoded by *CitLCYb1* was sufficient to convert all the lycopene produced by the *E. coli* XL1-BLUE-EIB cells into β-carotene. The activity of enzyme encoded by *CitLCYb2* was lower than that of *CitLCYb1* in *E. coli* cells. In the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with pGEX-6p-1-*CitLCYb2* from Satsuma mandarin and Valencia orange, both β-carotene and lycopene were detected (Fig. 4b). Similar results were also observed in Navel orange (Alquézar et al. 2009). So far, the roles of *LCYb1* and *LCYb2* in the biosynthesis of α-carotene are still unknown, although their functions in the biosynthesis of β-carotene have been characterized in some plants (Alquézar et al. 2009; Ampomah-Dwamena et al. 2009; Devitt et al. 2010). In the present study, we found that in the presence of *LCYe*, both the enzymes encoded by *CitLCYb1* and *CitLCYb2* could produce α-carotene *in E. coli* cells (Fig. 5). These results suggested that the *CitLCYb1* and *CitLCYb2* had similar functions: both their enzymes participated in the formation

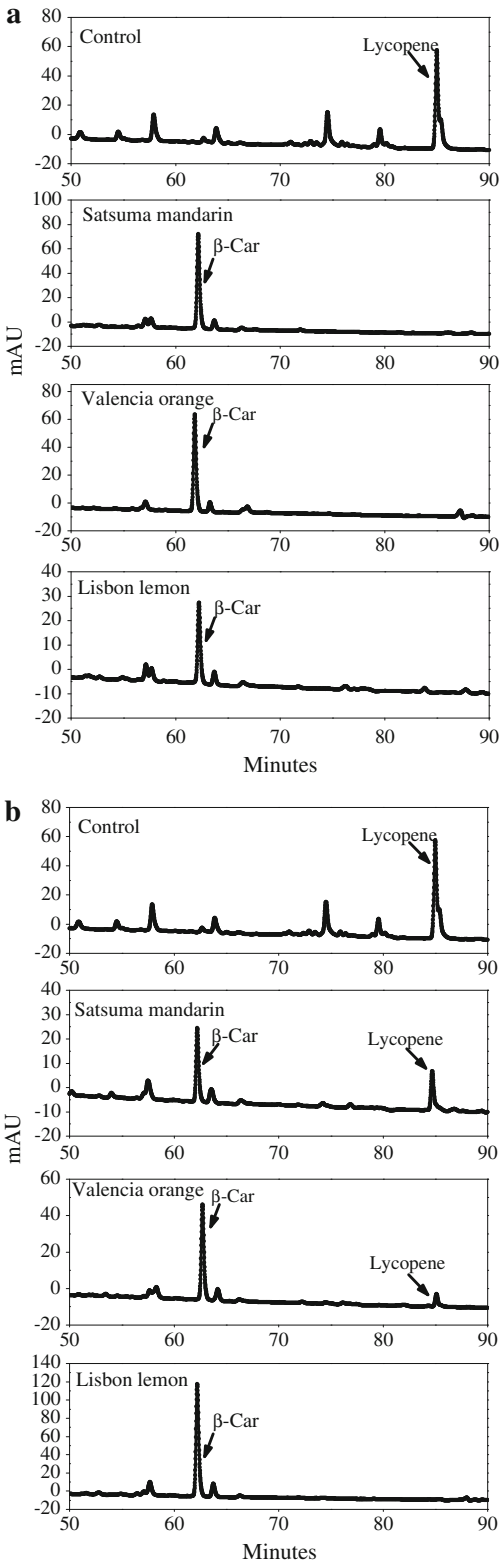

Fig. 4 HPLC analysis of carotenoids in *E. coli* XL1-BLUE-EIB cells transformed with pGEX-6p-1-*CitLCYb1* (**a**) and pGEX-6p-1-*CitLCYb2* (**b**) from Satsuma mandarin, Valencia orange and Lisbon lemon. Carotenoids extracted from the suspension cultures of *E. coli* XL1-BLUE-EIB cells with pGEX-6p-1 (empty vector) were used as a control. *β-Car* β-carotene

of β-carotene, and when they were co-expressed with *CitLCYe*, α-carotene could be produced from lycopene in *E. coli* cells.

It has reported that there existed two different alleles of *LCYb2*: *β-LCY2a* was isolated from Navel orange encoding a functional lycopene β-cyclase; *β-LCY2b* was isolated from red-fleshed Star Rugy grapefruit encoding a protein with almost null activity (Alquézar et al. 2009). *β-LCY2a* and *β-LCY2b* shared 96 % identity at the amino acid level with only 16 amino acid changes. Alquézar et al. (2009) predicted that slight alterations in the sequence of *β-LCY2b* caused the loss of the activity in Star Rugy grape fruit. In the present study, the 16 different amino acids were compared among *β-LCY2a*, *β-LCY2b* and *CitLCYb2* from three citrus varieties, Satsuma mandarin, Valencia orange and Lisbon lemon (Supplemental Table S1). The results showed that the sequence of *CitLCYb2* from the three citrus varieties was similar to that of *β-LCY2b*. However, functional assays showed that *CitLCYb2* from the three citrus varieties could convert lycopene into β-carotene in *E. coli* XL1-BLUE-EIB cells. Thus, the amino acid changes in *CitLCYb2* did not seem to affect its activity in Satsuma mandarin, Valencia orange or Lisbon lemon. Devitt et al. (2010) found that a TT insertion at 881 in the *lcy-β2* gene was responsible for the inactivation of chromoplast-specific lycopene β-cyclase and led to the accumulation of lycopene in the fruit of red-fleshed papaya.

Changes in the expression of *CitLCYb1* and *CitLCYb2* in the three citrus varieties during the ripening process

In citrus fruits, β,ε-carotenoids, such as α-carotene and lutein, accumulate with the high expression of *CitLCYe* during the green stage in the flavedo (Kato et al. 2004; Zhang et al. 2012). In the present study, we found that during the green stage the expression of *CitLCYb1*and *CitLCYe* increased significantly, while that of *CitLCYb2* remained low in the flavedo of Satsuma mandarin, Valencia orange and Lisbon lemon during the green stage (Fig. 6). Although both the enzymes of *CitLCYb1* and *CitLCYb2* could convert lycopene to α-carotene in *E. coli* cells, the extremely low gene expression level of *CitLCYb2* during the green stage in the flavedo indicated that *CitLCYb2* did not participate in the formation of α-carotene in the flavedo of citrus fruits. These results corroborate the previous observation that *LCYb2* was chromoplast-specific expression, and expressed exclusively in flowers and at the breaker-stage of fruit (Alquézar et al. 2009; Dalal et al. 2010; Mendes et al. 2011). Dalal et al. (2010) reported that the promoter of *ShCYC-B*, which was developmentally regulated and its expression was up-regulated in chromoplast-rich flowers and fruits, might play an important role in the regulation of the expression of *ShCYC-B* and the carotenoid contents in flowers and fruits.

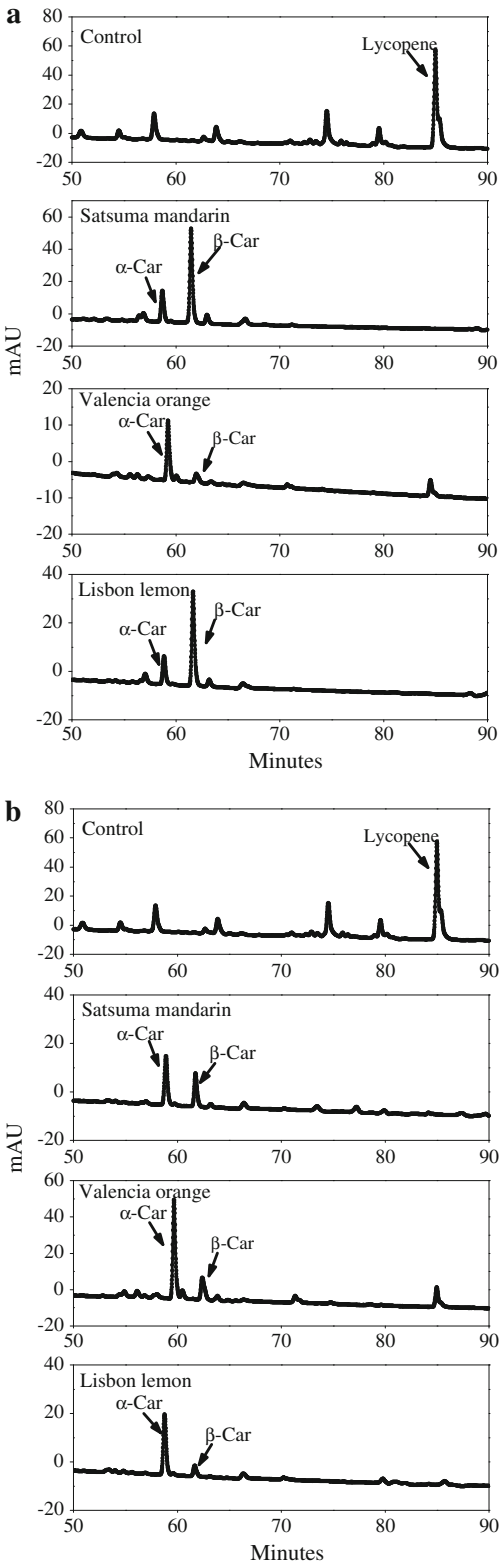

Fig. 5 HPLC analysis of carotenoids in *E. coli* XL1-BLUE-EIB cells transformed with pGEX-6p-1-*CitLCYb1*+*CitLCYe* (**a**) and pGEX-6p-1-*CitLCYb2*+*CitLCYe* (**b**) from Satsuma mandarin, Valencia orange and Lisbon lemon. Carotenoids extracted from the suspension cultures of *E. coli* XL1-BLUE-EIB cells with pGEX-6p-1 (empty vector) were used as a control. *α-Car* α-carotene, *β-car* β-carotene

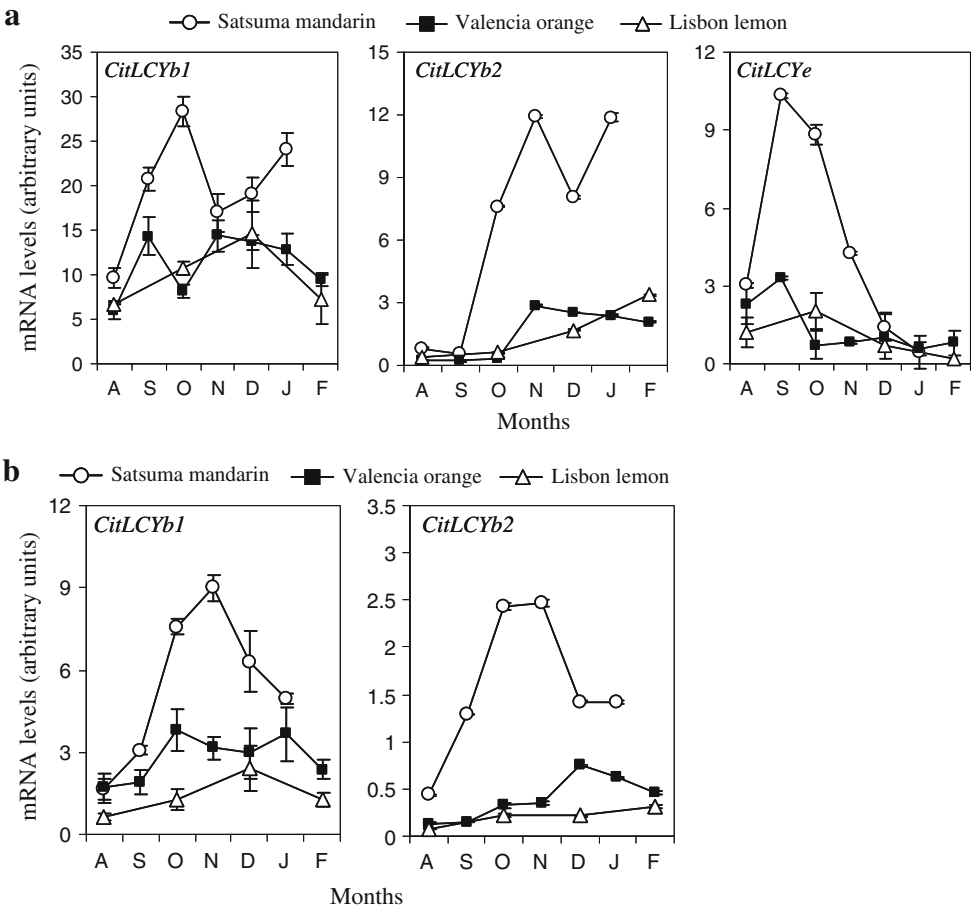

Fig. 6 Expression of *CitLCYb1*, *CitLCYb2* and *CitLCYe* in the flavedo (**a**) and expression of *CitLCYb1* and *CitLCYb2* in juice sacs (**b**). The mRNA levels were analyzed by TaqMan real-time quantitative RT-PCR. Real-time RT-PCR amplification of 18S rRNA was used to normalize the expression of the genes under identical conditions. The results shown are the mean ± SE for triplicate samples. *Some error bars and symbols* are hidden by symbols. *A* August, *S* September, *O* October, *N* November, *D* December, *J* January, *F* February

After the green stage, the carotenoid biosynthetic pathway changes from *β*,*ε*-carotenoid synthesis to *β*,*β*-carotenoid synthesis in citrus fruits (Kato et al. 2004; Zhang et al. 2012). In this study, the results showed that the expression of *CitLCYb1* and *CitLCYb2* increased to a maximum, while that of *CitLCYe* decreased significantly to a low level during the orange stage. The high expression of *CitLCYb1* and *CitLCYb2* led to the accumulation of *β*,*β*-carotenoids in the flavedo of Satsuma mandarin, Valencia orange and Lisbon lemon.

In the juice sacs, carotenoid biosynthetic pathway changing from *β*,*ε*-carotenoid synthesis to *β*,*β*-carotenoid synthesis occurred before August (Ikoma et al. 2001; Kato et al. 2004). Thus, the expression of *CitLCYe* was extremely low and almost undetectable in the juice sacs in both the green and orange stages in Satsuma mandarin, Valencia orange and Lisbon lemon. The expression of *CitLCYb1* and *CitLCYb2* increased rapidly in the green stage, and reached a maximum in the orange stage in the juice sacs of the three citrus varieties (Fig. 6). The increases in the expression of *CitLCYb1* and *CitLCYb2* resulted in a massive accumulation of *β*,*β*-xanthophylls, such as *β*-cryptoxanthin and 9-*cis*-violaxanthin, in the juice sacs of Satsuma mandarin, Valencia orange and Lisbon lemon.

The carotenoid composition in the juice sacs differed between Satsuma mandarin and Valencia orange. Satsuma mandarin accumulated *β*-cryptoxanthin, whereas Valencia orange mainly accumulated violaxanthin isomers (Kato et al. 2004). Previously, we found that higher levels of upstream synthesis genes (*CitPSY*, *CitPDS*, *CitZDS* and *CitLCYb1*), and lower levels of downstream synthesis genes (*CitHYb* and *CitZEP*) in Satsuma mandarin than those in Valencia orange led to the difference in carotenoid composition in these two citrus varieties (Kato et al. 2004; Zhang et al. 2012). In the present study, the expression level of *CitLCYb2* was much higher in Satsuma mandarin than that in Valencia orange during the ripening process, which indicated that the high expression level of *CitLCYb2* might be also closely related to the accumulation of *β*-cryptoxanthin in Satsuma mandarin (Fig. 6). In Lisbon lemon, *β*-cryptoxanthin was the major carotenoid, but its level was much lower than that in Satsuma mandarin (Kato et al. 2004). The present results showed that gene expression levels of *CitLCYb1* and *CitLCYb2* was much lower in Lisbon lemon than those in Satsuma mandarin. The extremely low expression of *CitLCYb1* and *CitLCYb2* might lead to the low content of *β*-cryptoxanthin in Lisbon

lemon. These results suggested that *CitLCYb1* and *CitLCYb2* played an important role in determining the profiles of carotenoids in citrus fruits.

In conclusion, in the present study two LCYb genes (*CitLCYb1* and *CitLCYb2*) were isolated from Satsuma mandarin, Valencia orange and Lisbon lemon. The results showed that *CitLCYb1* and *CitLCYb2* shared high identity at the amino acid level among the three citrus varieties. In Satsuma mandarin, the proteins encoded by *CitLCYb1* and *CitLCYb2* without the transit peptide had quite a similar secondary structure. Moreover, functional analysis showed that both the enzymes of *CitLCYb1* and *CitLCYb2* participated in the formation of β-carotene, and when they were co-expressed with *CitLCYe*, α-carotene could be produced from lycopene in *E. coli* cells. However, although *CitLCYb2* could convert lycopene to α-carotene, its extremely low expression level during the green stage in the flavedo indicated that *CitLCYb2* did not participate in the formation of α-carotene in the flavedo of citrus fruits. Moreover, the high expression levels of *CitLCYb1* and *CitLCYb2* during the orange stage played an important role in the accumulation of β,β-xanthophylls in citrus fruits.

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