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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 expoxidase
α-Car	α-Carotene
β-Car	β-Carotene

Introduction

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

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

169 harboring *CitLCYb1* or *CitLCYb2*. The recombinant plasmids constructed in the
170 present study were designated as: pGEX-6p-1-*CitLCYb1*, pGEX-6p-1-*CitLCYb2*,
171 pGEX-6p-1-*CitLCYb1*+*CitLCYe* and pGEX-6p-1-*CitLCYb2*+*CitLCYe*. The four
172 recombinant plasmids were transformed into lycopene-accumulating *E. coli*
173 XL1-Blue cells harboring a lycopene biosynthetic plasmid pACCRT-EIB.
174 (Misawa and Shimada 1997). The *E. coli* XL1-BLUE-EIB cells with
175 pGEX-6p-1 (empty vector) were used as a control. The transformants were
176 plated in LB medium supplemented with chloramphenicol (50 μg ml^{-1}) and
177 carbenicillin (50 μg ml^{-1}), and incubated at 37 ˚C for 20 h. The colonies were
178 incubated in 100 ml of 2×YT medium (l^{-1} 16g tryptone, 10 g yeast extract, and
179 5g NaCl) with chloramphenicol (50 μg ml^{-1}) and carbenicillin (50 μg ml^{-1}) at 37
180 ˚C for 16 h. Then, 2 ml of culture solution was inoculated into 200 ml of 2×YT
181 medium with chloramphenicol (50 μg ml^{-1}) and carbenicillin (50 μg ml^{-1}). After
182 cultured for 8h at 27 ˚C, 200 μl of 0.1 M isopropyl β-D-thiogalactoside was
183 added and the cells were cultured overnight at 27 ˚C.
184
185 Extraction and determination of carotenoids
186
187 Cultures of *E. coli* cells were centrifuged at 5000 g for 10 min and the
188 bacterial pellet was washed twice with Tris-HCl (pH 8.0). The pellet was dried
189 using vacuum freeze drying and stored at -20 ˚C until HPLC analysis. The
190 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

213 Mix (Applied Biosystems) using ABI PRISM 7300 (Applied Biosystems)
214 according to the manufacture's instructions. Each reaction contained 900 nM of
215 the primers, 250 nM of the TaqMan MGB Probe, and template cDNA. The
216 thermal cycling conditions were 95 ˚C for 10 min followed by 40 cycles of 95
217 ˚C for 15 s and 60 ˚C for 60 s. The levels of gene expression were analyzed with
218 ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems)
219 and normalized with the results of 18S ribosomal RNA. Real-time quantitative
220 RT-PCR was performed in three replicates for each sample.
221
222 **Results**
223
224 Isolation and sequence analysis of *CitLCYb1* and *CitLCYb2*
225
226 Two lycopene β-cyclase genes (*CitLCYb1* and *CitLCYb2*) were isolated from
227 three citrus varieties, Satsuma mandarin (accession no. AB114652 and
228 AB719392), Valencia orange (accession no. AB114660 and AB719393) and
229 Lisbon lemon (accession no. AB114668 and AB719394) and their nucleotide
230 and amino acid sequences were analyzed using the Genetyx Analysis Program.
231 As shown in Fig. 2, *CitLCYb1* and *CitLCYb2* showed high homology at the
232 amino acid level among Satsuma mandarin, Valencia orange and Lisbon lemon.
233 The identities of *CitLCYb1* and *CitLCYb2* between Satsuma mandarin and
234 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 kD. The nucleotide sequence of *CitLCYb2* contained 1,506 bp, encoding a putative protein of 502 amino acids with a calculated molecular mass of 56 kD. 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 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*

279 XL1-BLUE-EIB cells transformed with plasmids pGEX-6p-1-*CitLCYb2* from
280 Satsuma mandarin and Valencia orange, a small peak of lycopene was detected.
281 In the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with
282 plasmids pGEX-6p-1-*CitLCYb1*+*CitLCYe* from Satsuma mandarin, Valencia
283 orange and Lisbon lemon, peaks of β-carotene and α-carotene were observed
284 (Fig. 5a). The content of β-carotene was higher than that of α-carotene in the
285 extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids
286 pGEX-6p-1-*CitLCYb1*+*CitLCYe* from Satsuma mandarin and Lisbon lemon. In
287 the extract solution of *E. coli* XL1-BLUE-EIB cells transformed with plasmids
288 pGEX-6p-1-*CitLCYb2*+*CitLCYe* from the three citrus varieties, the peak of
289 α-carotene was observed, which was much higher than that of β-carotene (Fig.
290 5b).
291
292 Gene expression of *CitLCYb1*, *CitLCYb2* and *CitLCYe* in citrus fruits during
293 fruit ripening
294
295 According to the changes in the color of the flavedo, the ripening process of
296 the citrus fruits can be divided into two stages, a green stage and an orange stage.
297 The green stages in Satsuma mandarin, Valencia orange and Lisbon lemon were
298 from August to September, from August to October, and from August to October,
299 respectively (Kato et al. 2004, 2006).
300 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

345 similar structure and functional domains of LCY1 and LCY2 indicated that the
346 two LCYb genes might have similar functions in the biosynthesis of carotenoids
347 in citrus fruits.
348
349 Functional analysis of *CitLCYb1* and *CitLCYb2* in *E. coli* cells
350
351 The cyclization of lycopene, a key branch point in the pathway of carotenoid
352 biosynthesis in citrus fruits, involves two lycopene cyclases (LCY), β-cyclase
353 (LCYb) and ε-cyclase (LCYe) (Cunningham et al. 1996; Bai et al. 2009). In the
354 present study, functional analysis showed that in the extract solution of *E. coli*
355 XL1-BLUE-EIB cells transformed with pGEX-6p-1-*CitLCYb1* from the three
356 citrus varieties, only β-carotene was observed, the peak of lycopene having
357 disappeared completely (Fig. 4a). These results suggested that the enzyme
358 encoded by *CitLCYb1* was sufficient to convert all the lycopene produced by the
359 *E. coli* XL1-BLUE-EIB cells into β-carotene. The activity of enzyme encoded
360 by *CitLCYb2* was lower than that of *CitLCYb1* in *E. coli* cells. In the extract
361 solution of *E. coli* XL1-BLUE-EIB cells transformed with
362 pGEX-6p-1-*CitLCYb2* from Satsuma mandarin and Valencia orange, both
363 β-carotene and lycopene were detected (Fig. 4b). Similar results were also
364 observed in Navel orange (Alquézar et al. 2009). So far, the roles of *LCYb1* and
365 *LCYb2* in the biosynthesis of α-carotene are still unknown, although their
366 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 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

389 insertion at 881 in the *lcy*-β2 gene was responsible for the inactivation of
390 chromoplast-specific lycopene β-cyclase and led to the accumulation of
391 lycopene in the fruit of red-fleshed papaya.
392
393 Changes in the expression of *CitLCYb1* and *CitLCYb2* in the three citrus
394 varieties during the ripening process
395
396 In citrus fruits, β,ε-carotenoids, such as α-carotene and lutein, accumulate
397 with the high expression of *CitLCYe* during the green stage in the flavedo (Kato
398 et al. 2004; Zhang et al. 2012). In the present study, we found that during the
399 green stage the expression of *CitLCYb1*and *CitLCYe* increased significantly,
400 while that of *CitLCYb2* remained low in the flavedo of Satsuma mandarin,
401 Valencia orange and Lisbon lemon during the green stage (Fig. 6). Although
402 both the enzymes of *CitLCYb1* and *CitLCYb2* could convert lycopene to
403 α-carotene in *E. coli* cells, the extremely low gene expression level of *CitLCYb2*
404 during the green stage in the flavedo indicated that *CitLCYb2* did not participate
405 in the formation of α-carotene in the flavedo of citrus fruits. These results
406 corroborate the previous observation that *LCYb2* was chromoplast-specific
407 expression, and expressed exclusively in flowers and at the breaker-stage of fruit
408 (Alquézar et al. 2009; Dalal et al. 2010; Mendes et al. 2011). Dalal et al. (2010)
409 reported that the promoter of *ShCYC-B*, which was developmentally regulated
410 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. 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,

433 whereas Valencia orange mainly accumulated violaxanthin isomers (Kato et al.
434 2004). Previously, we found that higher levels of upstream synthesis genes
435 (*CitPSY*, *CitPDS*, *CitZDS* and *CitLCYb1*), and lower levels of downstream
436 synthesis genes (*CitHYb* and *CitZEP*) in Satsuma mandarin than those in
437 Valencia orange led to the difference in carotenoid composition in these two
438 citrus varieties (Kato et al. 2004; Zhang et al. 2012). In the present study, the
439 expression level of *CitLCYb2* was much higher in Satsuma mandarin than that in
440 Valencia orange during the ripening process, which indicated that the high
441 expression level of *CitLCYb2* might be also closely related to the accumulation
442 of β-cryptoxanthin in Satsuma mandarin (Fig. 6). In Lisbon lemon,
443 β-cryptoxanthin was the major carotenoid, but its level was much lower than
444 that in Satsuma mandarin (Kato et al. 2004). The present results showed that
445 gene expression levels of *CitLCYb1* and *CitLCYb2* was much lower in Lisbon
446 lemon than those in Satsuma mandarin. The extremely low expression of
447 *CitLCYb1* and *CitLCYb2* might lead to the low content of β-cryptoxanthin in
448 Lisbon lemon. These results suggested that *CitLCYb1* and *CitLCYb2* played an
449 important role in determining the profiles of carotenoids in citrus fruits.

450 In conclusion, in the present study two LCYb genes (*CitLCYb1* and
451 *CitLCYb2*) were isolated from Satsuma mandarin, Valencia orange and Lisbon
452 lemon. The results showed that *CitLCYb1* and *CitLCYb2* shared high identity at
453 the amino acid level among the three citrus varieties. In Satsuma mandarin, the
454 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.

Acknowledgements

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Figure legends

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 expoxidase.

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).

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).

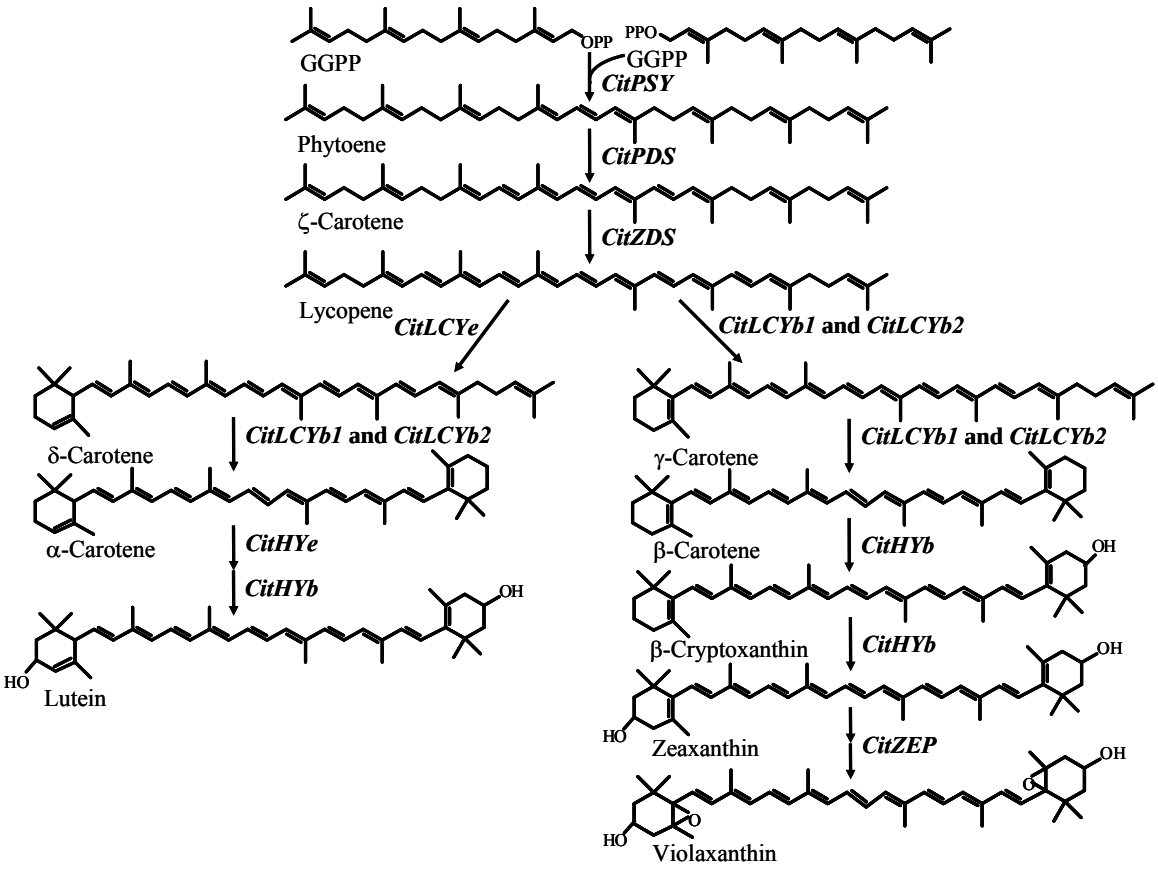
Fig. 4 HPLC analysis of carotenoids in *E. coli* XL1-BLUE-EIB cells transformed with pGEX-6p-1-*CitLCYb1* (**a**) and pGEX-6p-1-*CitLCYb2* (**b**) from Satsuma mandarin, Valencia orange and Lisbon lemon. Carotenoids extracted from the suspension cultures of *E. coli* XL1-BLUE-EIB cells with pGEX-6p-1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

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.

Fig. 1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δ-Carotene
CitLCYb1 and CitLCYb2
α-Carotene
CitHYe
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HO
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γ-Carotene
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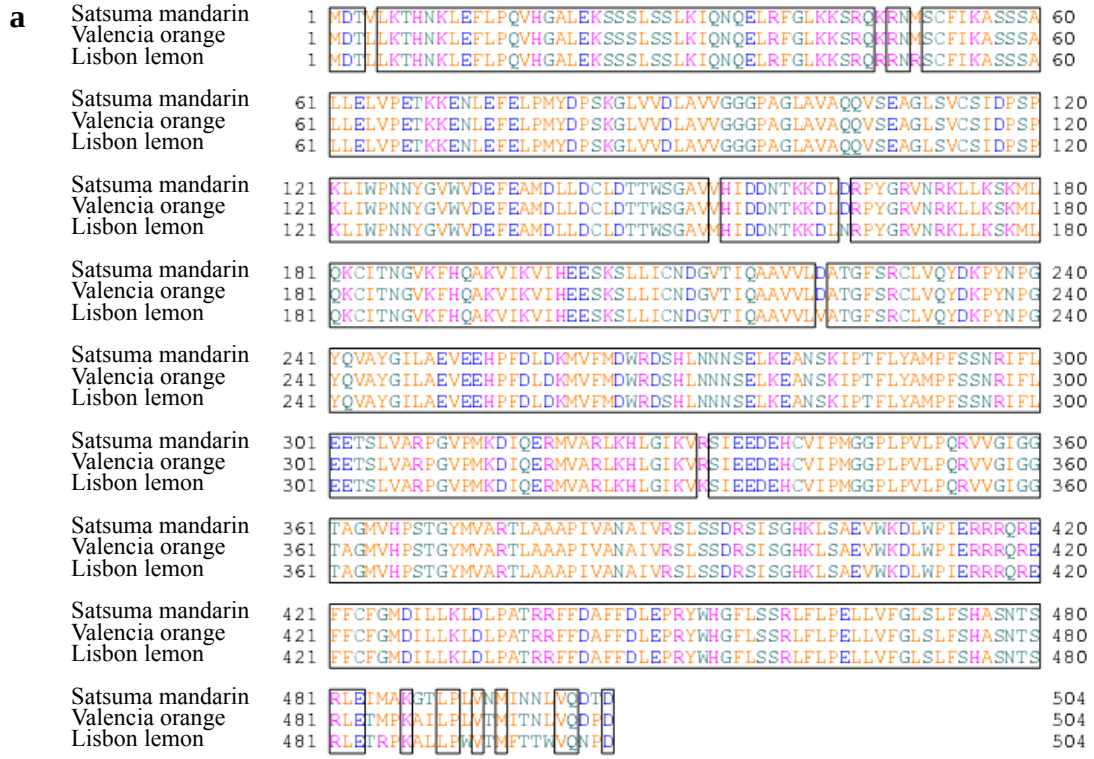
Fig. 2

a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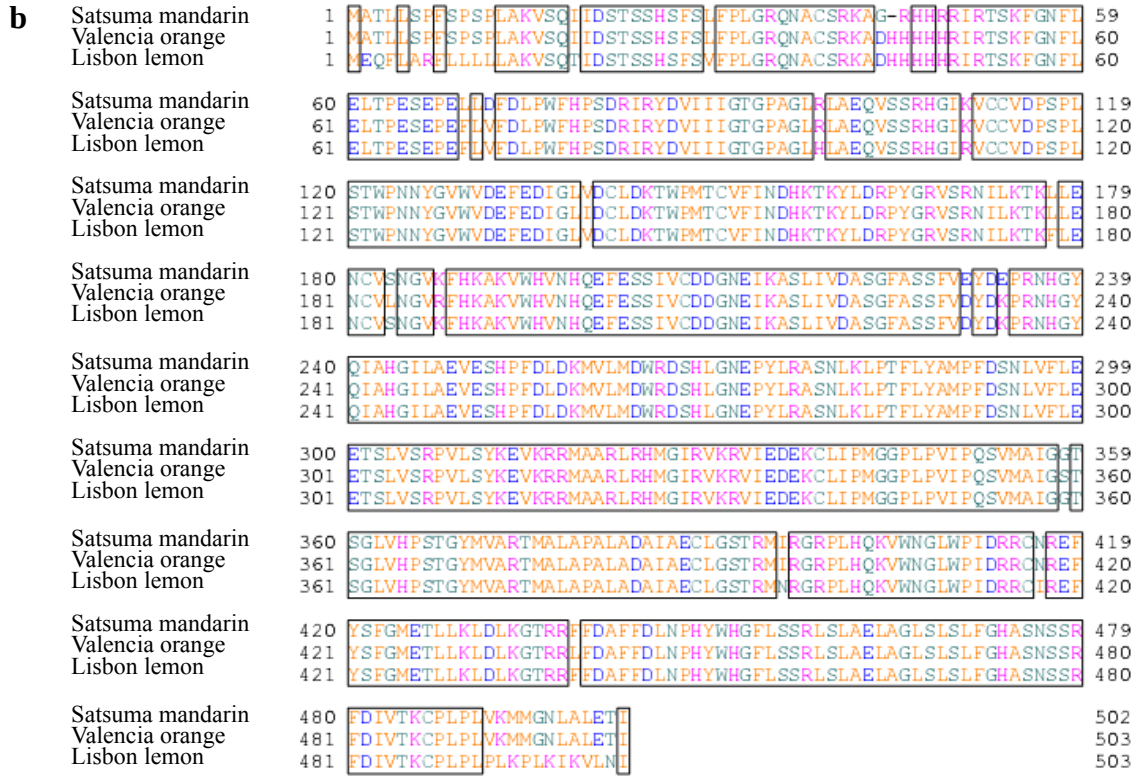
Valencia orange
Lisbon lemon

b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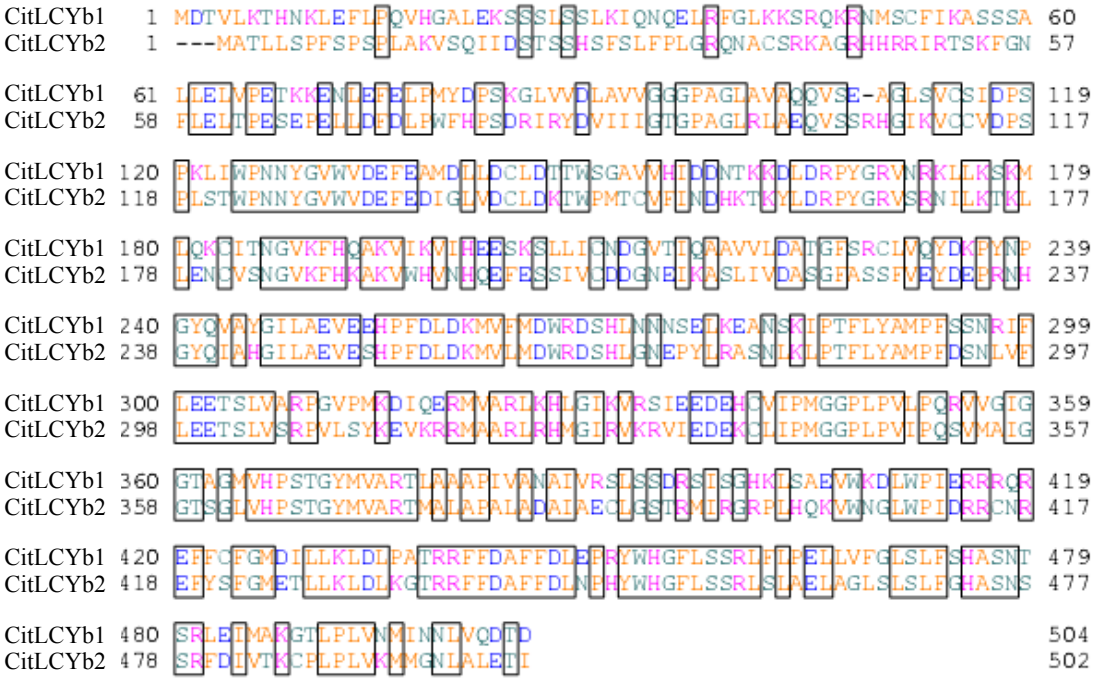
Lisbon lemon

Fig. 3


CitLCYb1 1 MDTVLKTHNKLEFLPQVHGALEKSSSLSSLKIQNQELRFGLKKSRQKRNMSCFIKASSSA 60
CitLCYb2 1 ---MATLLSPFSPSPLAKVSQIIDSTSSHSFSLFPLGRQNACSRKAGRHHRRIRTSKFGN 57
CitLCYb1 61 LLELVPETKKENLEFELPMYDPSKGLVVDLAVVGGGPAGLAVAQQVSE-AGLSVCSIDPS 119
CitLCYb2 58 FLELTPESEPELLDFDLPWFHPSDRIRYDVIIIGTGPAGLRLAEQVSSRHGIKVCCVDPS 117
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CitLCYb2 118 PLSTWPNNYGVWVDEFEDIGLVDCLDKTWPMTCVFINDHKTKYLDRPYGRVSRNILKTKL 177
CitLCYb1 180 LQKCITNGVKFHQAKVIKVIHEESKSLLICNDGVTIQAAVVLDATGFSRCLVQYDKPYNP 239
CitLCYb2 178 LENCVSNGVKFHKAKVWHVNHQEFESSIVCDDGNEIKASLIVDASGFASSFVEYDEPRNH 237
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CitLCYb2 238 GYQIAHGILAEVESHPFDLDKMVLMDWRDSHLGNEPYLRASNLKLPTFLYAMPFDSNLVF 297
CitLCYb1 300 LEETSLVARPGVPMKDIQERMVARLKHLGIKVRSIEEDEHCVIPMGGPLPVLPQRVVGIG 359
CitLCYb2 298 LEETSLVSRPVLSYKEVKRRMAARLRHMGIRVKRVIEDEKCLIPMGGPLPVIPQSVVAIG 357
CitLCYb1 360 GTAGMVHPSTGYMVARTLAAAPIVANAIVRSLSSDRSISGHKLSAEVWKDLWPIERRRQR 419
CitLCYb2 358 GTSGLVHPSTGYMVARTMALAPALADAIAECLGSTRMIRGRPLHQKVWNGLWPIDRRCNR 417
CitLCYb1 420 EFFCFGMDILLKLDLPATRRFFDAFFDLEPRYWHGFLSSRLFLPELLVFGLSLFSHASNT 479
CitLCYb2 418 EFYSFGMETLLKLDLKGTRRFFDAFFDLNPHYWHGFLSSRLSLAELAGLSLSLFGHASNS 477
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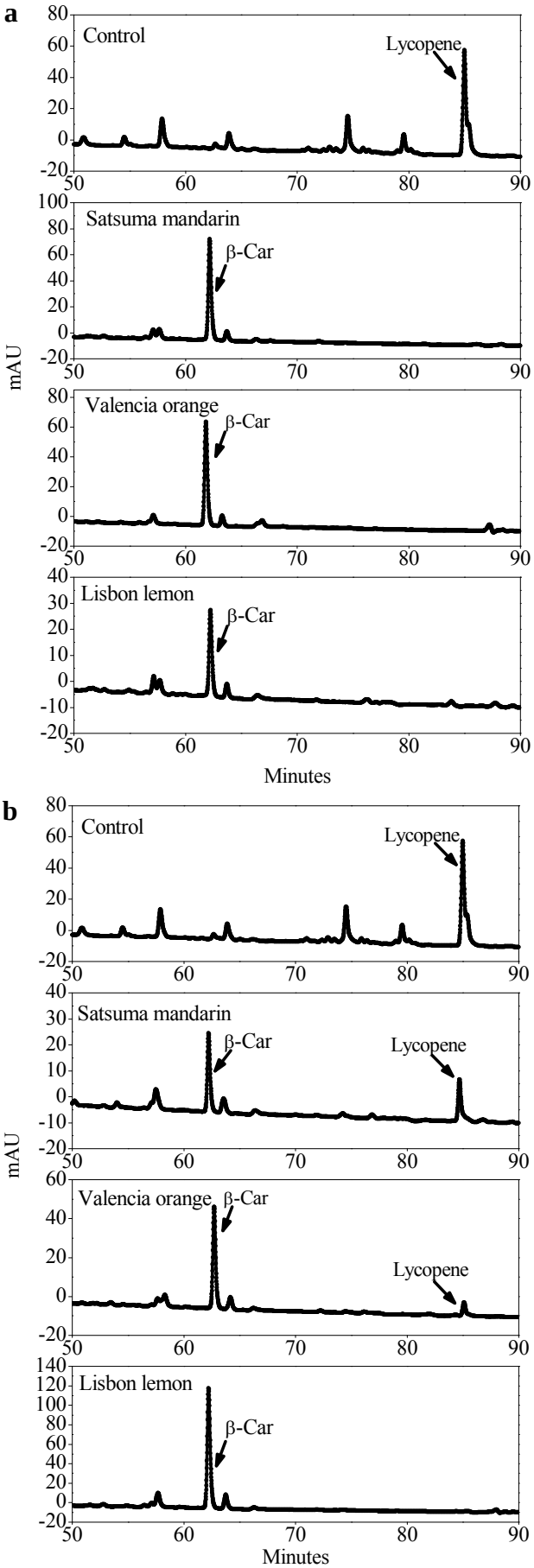
CitLCYb2 478 SRFDIVTKCPLPLVKMMGNLALETI 502

Fig. 4


a
Control
Lycopene
Satsuma mandarin
β-Car
Valencia orange
β-Car
Lisbon lemon
β-Car
mAU
Minutes
b
Control
Lycopene
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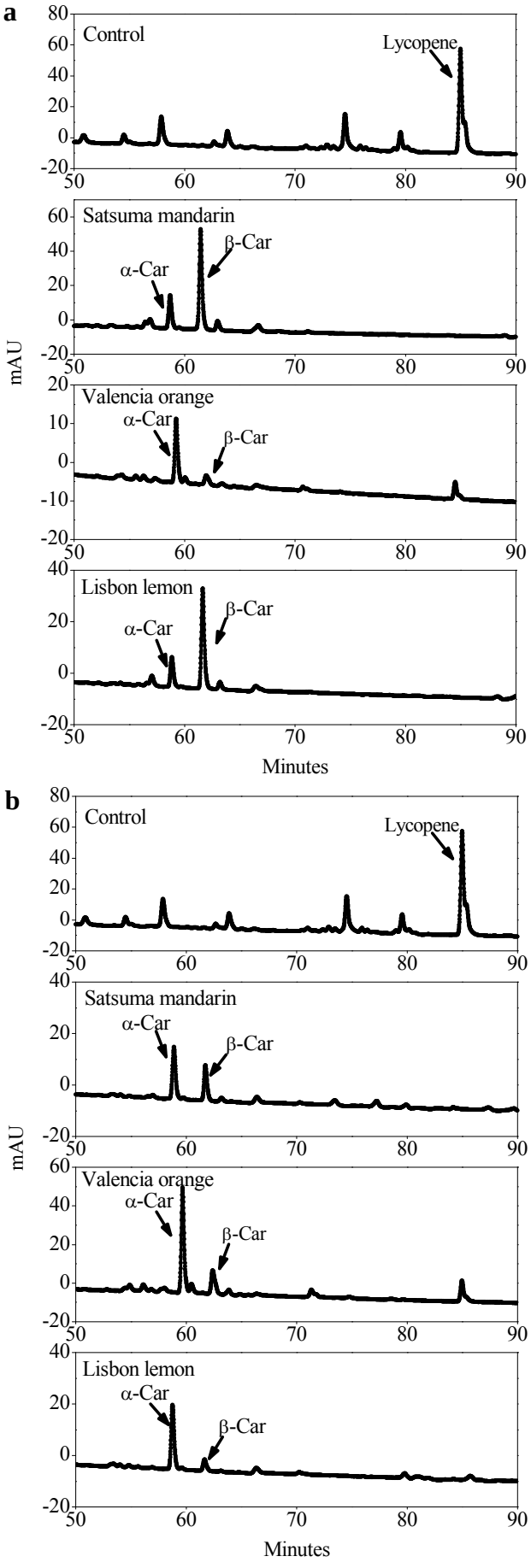
β-Car
Lycopene
Valencia orange
β-Car
Lycopene
Lisbon lemon
β-Car
mAU
Minutes

Fig. 5


a
Control
Lycopene
Satsuma mandarin
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β-Car
Valencia orange
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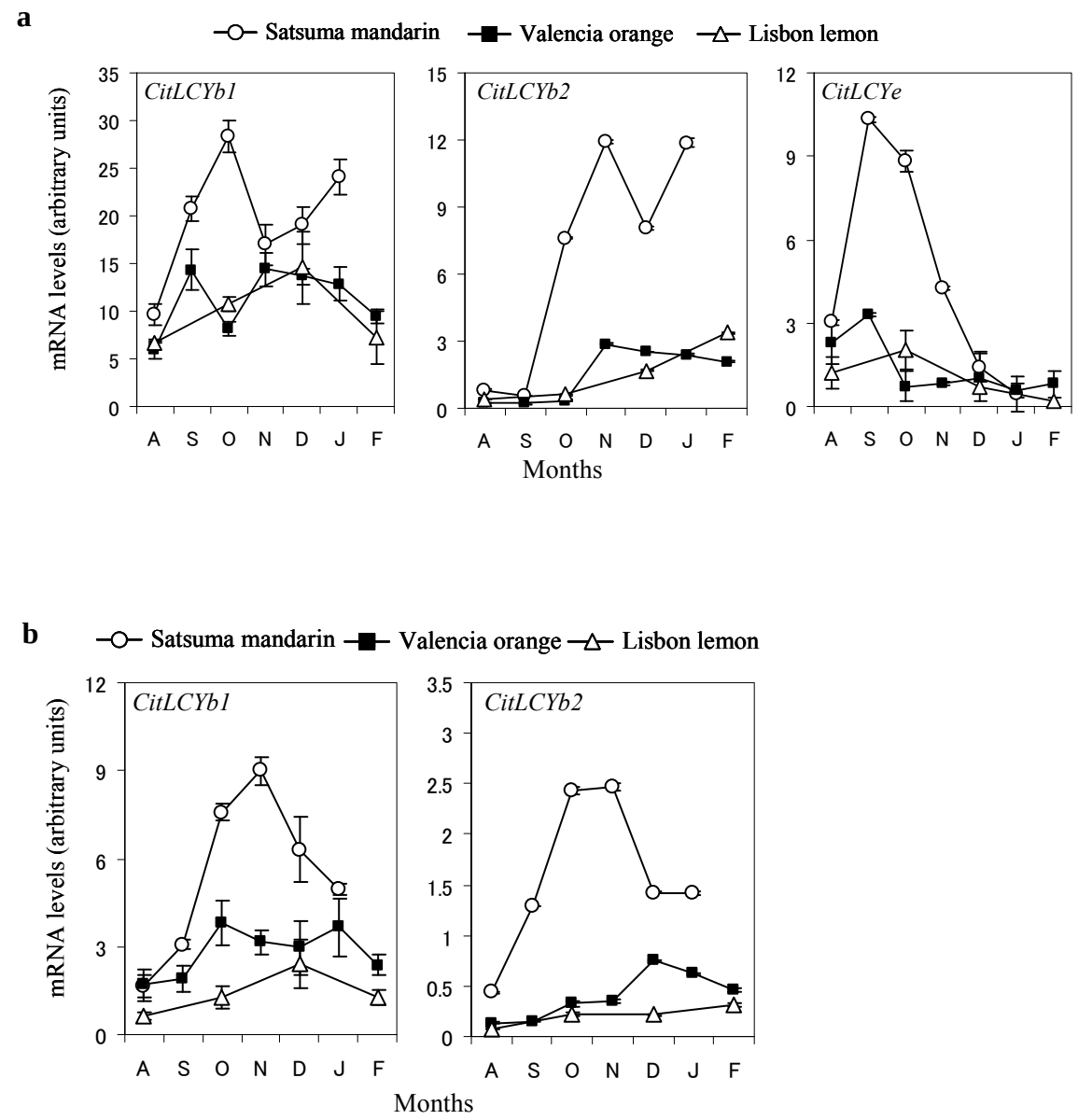
β-Car
Lisbon lemon
α-Car
β-Car
mAU
Minutes
b
Control
Lycopene
Satsuma mandarin
α-Car
β-Car
Valencia orange
α-Car
β-Car
Lisbon lemon
α-Car
β-Car
mAU
Minutes

Fig. 6


a
Satsuma mandarin
Valencia orange
Lisbon lemon
CitLCYb1
CitLCYb2
CitLCYe
mRNA levels (arbitrary units)
A S O N D J F
Months
b
Satsuma mandarin
Valencia orange
Lisbon lemon
CitLCYb1
CitLCYb2
mRNA levels (arbitrary units)
A S O N D J F
Months

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

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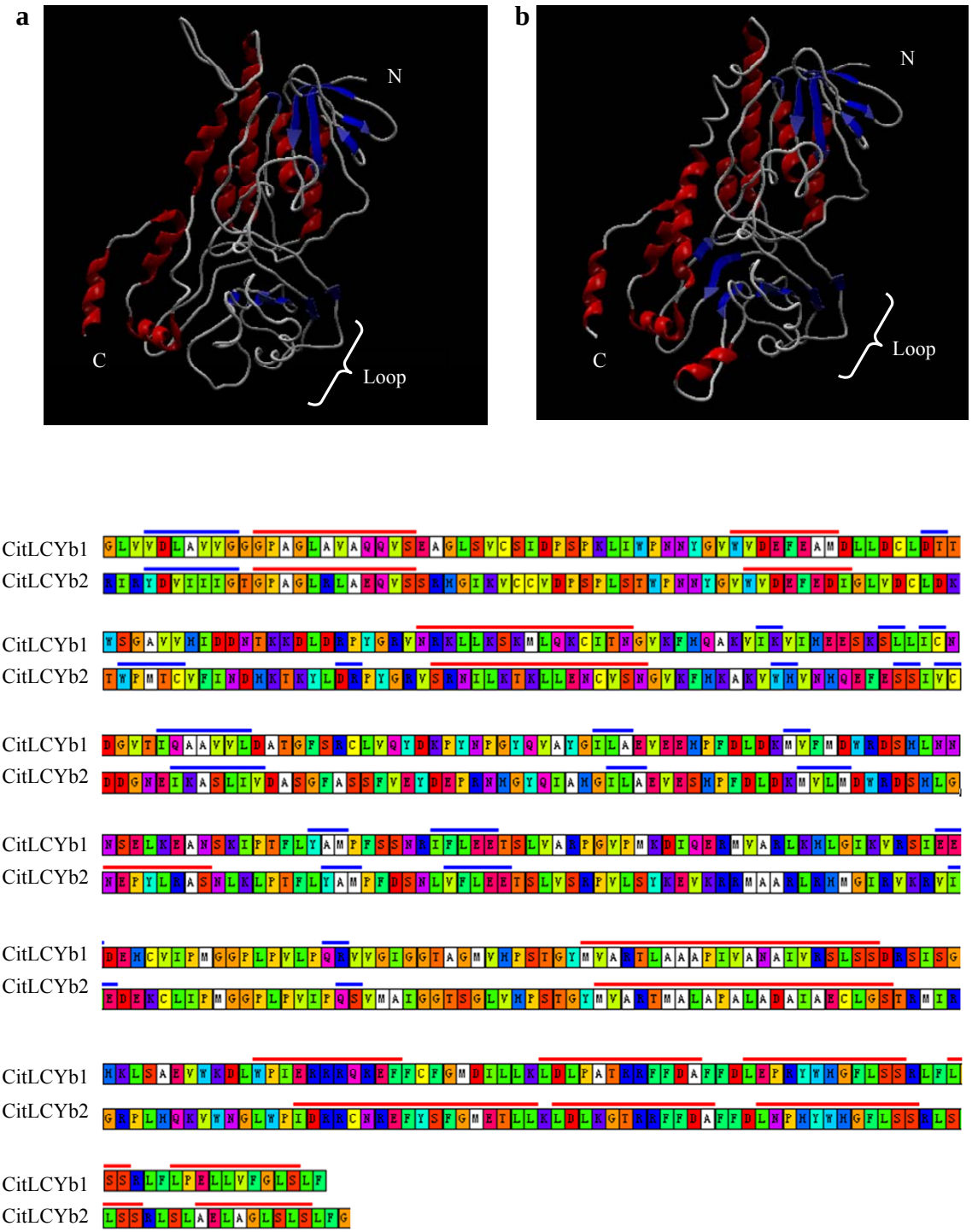
Supplemental Table S1 Changes in amino acid among the *CitLCYb2* and two *β-LCY* alleles.

Position	*CitLCYb2*	*CitLCYb2*	*CitLCYb2*	*β-LCY2a*	*β-LCY2b*
Amino acid	Satsuma mandarin	Valencia orange	Lisbon lemon	Novel orange	Star Ruby grapefruit
26	H	H	H	P	H
67	E	E	E	V	E
72	D	V	V	D	V
109	G	G	G	S	G
110	I	I	I	V	I
140	V	I	V	V	I
184	S	L	S	S	L
188	K	R	K	K	R
231	D	D	D	E	D
317	R	R	R	S	R
359	G	S	G	G	S
364	V	V	V	I	V
367	S	S	S	A	S
449	H	H	H	Y	H
481	F	F	F	L	F
489	L	L	L	V	L

CitLCYb2 was isolated from Satsuma mandarin, Valencia orange and Lisbon lemon, respectively. *β-LCY2a* was isolated from Navel orange; *β-LCY2b* was isolated from Star Ruby grapefruit (Alquézar et al., 2009).

Supplemental Figure S1 Structural comparison of proteins encoded by *CitLCYb1* and *CitLCYb2* in Satsuma mandarin. **a** Structure of the protein encoded by *CitLCYb1*. **b** Structure of the protein encoded by *CitLCYb2*. **c** Amino acid sequences of *CitLCYb1* and *CitLCYb2* showing the position of α-helixes (red lines) and β-strands (blue lines). The structure of the protein encoded by *CitLCYb1* and *CitLCYb2* was predicted by Swiss-Model server.

Fig. S1


a
N
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Loop
b
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C
Loop
c
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CitLCYb2
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