Expression and functional analysis of citrus carotene hydroxylases: unravelling the xanthophyll biosynthesis in citrus fruits

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Abstract

Background: Xanthophylls are oxygenated carotenoids and fulfill critical roles in plant growth and development. In plants, two different types of carotene hydroxylases, non-heme di-iron and heme-containing cytochrome P450, were reported to be involved in the biosynthesis of xanthophyll. Citrus fruits accumulate a high amount of xanthophyll, especially β,β-xanthophylls. To date, however, the roles of carotene hydroxylases in regulating xanthophyll content and composition have not been elucidated.

Results: In the present study, the roles of four carotene hydroxylase genes (CitHYb, CitCYP97A, CitCYP97B, and CitCYP97C) in the biosynthesis of xanthophyll in citrus fruits were investigated. Phylogenetic analysis showed that the four citrus carotene hydroxylases presented in four distinct clusters which have been identified in higher plants. CitHYb was a non-heme di-iron carotene hydroxylase, while CitCYP97A, CitCYP97B, and CitCYP97C were heme-containing cytochrome P450-type carotene hydroxylases. Gene expression results showed that the expression of CitHYb increased in the flavedo and juice sacs during the ripening process, which was well consistent with the accumulation of β,β-xanthophyll in citrus fruits. The expression of CitCYP97A and CitCYP97C increased with a peak in November, which might lead to an increase of lutein in the juice sacs during the ripening process. The expression level of CitCYP97B was much lower than that of CitHYb, CitCYP97A, and CitCYP97C in the juice sacs during the ripening process. Functional analysis showed that the CitHYb was able to catalyze the hydroxylation of the β-rings of β-carotene and α-carotene in Escherichia coli BL21 (DE3) cells. Meanwhile, when CitHYb was co-expressed with CitCYP97C, α-carotene was hydroxylated on the β-ring and ε-ring sequentially to produce lutein.

Conclusions: CitHYb was a key gene for β,β-xanthophyll biosynthesis in citrus fruits. CitCYP97C functioned as an ε-ring hydroxylase to produce lutein using zeinoxanthin as a substrate. The results will contribute to elucidating xanthophyll biosynthesis in citrus fruits, and provide new strategies to improve the nutritional and commercial qualities of citrus fruits.

Keywords: β-Cryptoxanthin, Flavedo, Juice sacs, Lutein, Satsuma mandarin

Background

Carotenoids are a diverse group of pigments widely distributed in nature that provide distinct colors to fruits and flowers, and fulfill critical roles in plant growth and development [1–4]. In nature, more than 700 carotenoids have been identified and divided into two groups: carotenes and xanthophylls. Carotenes are linear or cyclic hydrocarbons, and xanthophylls are oxygenated derivatives of carotenes, such as lutein, β-cryptoxanthin, zeaxanthin, and astaxanthin. In higher plants, xanthophylls play an important role in the photosynthesis and photoprotection. They are structural elements of the photosynthetic apparatus, and the xanthophyll cycle (lutein, zeaxanthin, and anthoxanthin) protects plants from the damage of high light irradiation by dissipating excess light energy [5–8]. In addition, xanthophylls can be oxidatively cleaved in a site-specific manner, producing different apocarotenoids with important metabolic functions, such as plant hormones, pigments, as well...
as aroma and scent compounds [9–13]. Xanthophylls are not only important to the plants themselves, but also beneficial to human health. Epidemiological studies suggested that xanthophylls, such as lutein, β-cryptoxanthin, and astaxanthin, were effective to prevent eye diseases, certain cancers and inflammation because of their high antioxidant activity [14–21].

In plants, two different types of carotene hydroxylases, non-heme di-iron carotene hydroxylase and heme-containing cytochrome P450-type carotene hydroxylase, are involved in the biosynthesis of xanthophyll. Non-heme di-iron carotene hydroxylase (also called BCH, HYD, or HYb) efficiently catalyzes the hydroxylation of the β-rings of β-carotene (Fig. 1). In some plants species, it has been reported that two members of non-heme di-iron carotene hydroxylase existed, which had similar functions but tissue-specific expression patterns [22–25]. In Arabidopsis, a double-null mutation of BCH1 and BCH2 led to a significant decrease of ββ-xanthophylls [24, 26]. Recently, three heme-containing cytochrome P450-type carotene hydroxylases (CYP97A3, CYP97B3, and CYP97C1) have been identified in Arabidopsis. As shown in Fig. 1, CYP97C1 encoded by the LUT1 locus in Arabidopsis is responsible for the ε-ring hydroxylation [27, 28]. CYP97C1 is a key enzyme for the biosynthesis of lutein, and its activity can not be replaced by other carotene hydroxylases. In tomato, up-regulation of CYP97C11 led to an increase in the content of lutein in leaves. In contrast, when CYP97C11 was down-regulated, lutein was almost absent (0.8 %) in tomato leaves [29]. CYP97A3 encoded by LUIT locus exhibits a major activity towards the β-ring of α-carotene, and a minor activity towards the β-rings of β-carotene in Arabidopsis [28, 30] (Fig. 1). Quinlan et al. [31] reported that OsCYP97A4 interacted with OsCYP97C2 in maize protoplasts, and the synergistic interaction between OsCYP97A4 and OsCYP97C2 drove the formation of lutein. Unlike CYP97A and CYP97C, the roles of CYP97B in the xanthophyll biosynthesis are still poorly studied. It has been suggested that CYP97B3 might be able to hydroxylate the β-rings of β-carotene and α-carotene in Arabidopsis [32, 33]. However, in the quadruple mutant (bch1, bc12, cyp97c1, and cyp97a3) that contained only CYP97B3, xanthophylls did not accumulated, indicating that CYP97B might not be an important enzyme for carotene hydroxylation [8, 24].

Citrus fruits accumulate a high amount of xanthophylls, especially ββ-xanthophylls, which account for up to 90 % of total carotenoids [34, 35]. In the previous studies, carotenoid metabolism has been extensively investigated in the fruits of different citrus varieties [11, 34–38]. Meanwhile, some key carotenoid metabolic genes have been isolated and their functions were deeply investigated in citrus fruits [9, 11, 12, 37, 39]. To date, however, the roles of carotene hydroxylases in regulating carotenoid content and composition are still unclear in citrus. In the present study, the changes in the expression of four carotene hydroxylase genes (CitHYb, CitCYP97A, CitCYP97B, and CitCYP97C) were investigated in the flavedo and juice sacs during the ripening process. In addition, to elucidate their roles in xanthophyll biosynthesis, functional analyses

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**Fig. 1** Xanthophyll biosynthetic pathway in Arabidopsis. LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; BCH1/2, β-ring hydroxylase1/2; CYP97A3, heme-containing cytochromes P450 monooxygenases A3; CYP97B3 heme-containing cytochromes P450 monooxygenases B3; CYP97C1, heme-containing cytochrome P450 monooxygenases C1; ZEP, zeaxanthin epoxidase.
of the four carotene hydroxylase genes were conducted in *Escherichia coli* cells accumulating with different carotenoids. The results present in this study will contribute to further elucidating the mechanism of carotenoid accumulation in citrus fruits, and provide new insights into enhancing the nutritional and commercial qualities of citrus fruits.

**Results**

**Isolation and characterization of carotene hydroxylase genes in citrus fruits**

In order to identify the carotene hydroxylase genes in citrus, we performed blast searches in the Citrus clementina v.10 and Citrus sinensis v 1.1 genome databases (http://www.phytozome.net/) using the sequences of Arabidopsis *BCH1*, *BCH2*, *CYP97A3*, *CYP97B3*, and *CYP97C1* as queries, respectively. Four carotene hydroxylase genes (*HYb*, *CYP97A*, *CYP97B*, and *CYP97C*) were identified in citrus genome database. In our previous study, *CitHYb* was isolated from Satsuma mandarin (Accession number: AB114653), while the information on *CYP97A*, *CYP97B*, and *CYP97C* in citrus fruits was completely unknown. In the present study, the full-length cDNAs of *CYP97A*, *CYP97B*, and *CYP97C* were isolated from Satsuma mandarin by RT-PCR using the primers designed within 5′ and 3′ UTRs according to the sequences obtained from the citrus genome database. The sequences of *CYP97A*, *CYP97B*, and *CYP97C* were named as *CitCYP97A*, *CitCYP97B*, and *CitCYP97C*, and submitted to the NCBI database (Accession numbers: *CitCYP97A*, LC143646; *CitCYP97B*, LC143647; *CitCYP97C*, LC143648). Phylogenetic analysis showed that the four citrus carotene hydroxylases presented in four distinct clusters which have been identified in higher plants (Fig. 2). CitHYb was a non-heme di-iron carotene hydroxylase, and its nucleotide sequence contained 936 bp, encoding a putative protein of 311 amino acids with a predicted molecular of 34.7 kDa. CitCYP97A, CitCYP97B, and CitCYP97C were heme-containing cytochrome P450-type carotene hydroxylases. The nucleotide sequence of *CitCYP97A* contained 1839 bp, and encoded a putative protein of 612 amino acids with a predicted molecular of 68.4 kDa. The nucleotide sequence of *CitCYP97B* contained 1749 bp, and encoded a putative protein of 582 amino acids with a predicted molecular of 65.2 kDa. The nucleotide sequence of *CitCYP97C* contained 1641 bp, and encoded a putative protein of 546 amino acids with

![Fig. 2 Phylogenetic analysis of carotene hydroxylases. The Neighbor-joining phylogenetic tree was constructed based on the alignment of the deduced amino acid sequences of carotene hydroxylases using MEGA6 software](image-url)
a predicted molecular of 61.6 kDa. A chloroplastic transit peptide with different lengths was predicted in the N-terminal region of the proteins of CitHYb (62 amino acids), CitCYP97A (37 amino acids), CitCYP97B (50 amino acids), and CitCYP97C (18 amino acids).

Changes in carotenoid contents and expression of carotene hydroxylase genes in the flavedo during the ripening process

In the present study, carotenoids were extracted from citrus fruits during the ripening process, and the changes in carotenoid content and composition were analyzed by HPLC. In the flavedo, the contents of β-carotene, α-carotene and lutein decreased rapidly from August, and then kept at a low level during the ripening process (Fig. 3a). The content of β-cryptoxanthin, the major carotenoid in Satsuma mandarin, increased significantly during the ripening process, and reached 49.7 μg g⁻¹ in December. In addition, the contents of zeaxanthin, all-trans-violaxanthin and cis-violaxanthin also gradually increased, and as a result β,β-xanthophyll (sum of β-cryptoxanthin, zeaxanthin, all-trans-violaxanthin, and cis-violaxanthin) accumulated massively during the ripening process (Fig. 3b). Gene expression results showed that the expression of CitHYb increased gradually from September, which was consistent with the accumulation of β,β-xanthophyll during the ripening process (Fig. 4a). The expression of CitCYP97A decreased rapidly to a low level in October, and then increased with a peak in November during the ripening process. The expression of CitCYP97C increased with two peaks in September and November, respectively. Similarly to CitCYP97C, the expression of CitCYP97B increased gradually with a peak in September.

Changes in carotenoid contents and expression of carotene hydroxylase genes in the juice sacs during the ripening process

In the juice sacs, the content of β-carotene decreased rapidly to an extremely low level in October, while the contents of β-cryptoxanthin and zeaxanthin increased significantly during the ripening process (Fig. 3c). The contents of α-carotene and lutein increased gradually in the juice sacs during the ripening process. Gene expression results showed that the expression of CitHYb increased in the juice sacs during the ripening process, which was in parallel with the accumulation of β,β-xanthophyll (Fig. 4b). The expression of CitCYP97A, CitCYP97B, and CitCYP97C increased with a peak in October and November, respectively. In addition, the expression level of CitCYP97B was much lower than that of CitCYP97A and CitCYP97C in the juice sacs during the ripening process (Fig. 4b).

Functional analysis of carotene hydroxylase genes in E. coli cells

In the present study, the cDNAs of CitHYb, CitCYP97A, CitCYP97B, and CitCYP97C without transcript peptides were cloned into pRSF-2 Ek/LIC vector, respectively. The four recombinant plasmids were transformed into β-carotene-accumulating E. coli BL21 (DE3) cells, as well as α-carotene- and β-carotene-accumulating E. coli BL21 (DE3) cells, respectively. Carotenoids were extracted from bacteria and analyzed by HPLC. When CitHYb was expressed in the β-carotene-accumulating E. coli BL21 (DE3) cells, the peaks of β-cryptoxanthin and zeaxanthin were observed (Fig. 5b). When CitHYb was expressed in the α-carotene- and β-carotene-accumulating E. coli BL21 (DE3) cells, a monohydroxylated intermediate, zeinoxanthin, was also detected, except for β-cryptoxanthin and zeaxanthin (Fig. 6b).

To further investigate the functions of carotene hydroxylase genes of citrus, we co-transformed CitCYP97C with CitHYb, CitCYP97A, and CitCYP97B, respectively. The recombinant plasmids were expressed in the α-carotene- and β-carotene-accumulating E. coli BL21 (DE3) cells. As shown in Fig. 7b, when CitHYb and CitCYP97C were co-expressed, the monohydroxylated zeinoxanthin, which was produced by CitHYb, was further converted to lutein by CitCYP97C. However, when CitCYP97A and CitCYP97C were co-expressed or CitCYP97B and CitCYP97C were co-expressed, no hydroxylated carotene was detected in the α-carotene- and β-carotene-accumulating E. coli BL21 (DE3) cells (Figs. 5c, d, e and 6c, d, e).

Discussion

Isolation and characterization of carotene hydroxylase genes in citrus fruits

Carotene hydroxylases were the key enzymes responsible for xanthophyll biosynthesis in plants. Two different types of carotene hydroxylases, non-heme di-iron carotene hydroxylase and heme-containing cytochrome P450-type carotene hydroxylase, have been identified in higher plants. In the present study, the roles of four carotene hydroxylase genes (CitHYb, CitCYP97A, CitCYP97B, and CitCYP97C) in regulating xanthophylls biosynthesis were investigated in citrus fruits. As shown in Fig. 2, the four carotene hydroxylase genes were clustered in distinct groups. CitHYb was a non-heme di-iron carotene hydroxylase, while CitCYP97A, CitCYP97B, and CitCYP97C were heme-containing cytochrome P450-type carotene hydroxylases. It has been reported that two or more HYb genes existed in some plant species, such as pepper, tomato, and
Arabidopsis [23, 25, 40–42]. However, only one HYb was identified and isolated from citrus fruits, and it showed approximately 70% identities to Arabidopsis BCH1 and BCH2 at the amino acid level. In the previous studies, the changes in the expression of HYb were extensively investigated in citrus fruits during the ripening process and under different environmental conditions [11, 34, 35, 43, 44]. In the juice sacs, different expression levels of CitHYb led to distinct carotenoid compositions between Satsuma mandarin and Valencia orange [34]. In contrast...
**Fig. 5** HPLC analysis of carotenoids in β-carotene-accumulating *E. coli* BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitHYb (b), pRSF-2 Ek/LIC-CitCYP97A (c), pRSF-2 Ek/LIC-CitCYP97B (d), and pRSF-2 Ek/LIC-CitCYP97C (e). Carotenoids extracted from the suspension cultures of β-carotene-accumulating *E. coli* BL21 (DE3) cells with pRSF-2 (empty vector) were used as control (a). β-Car, β-carotene; β-Cry, β-cryptoxanthin; Zea, zeaxanthin.
to CitHYb, the roles of heme-containing cytochrome P450-type carotene hydroxylases in regulating carotenoid accumulation in citrus fruits are completely unknown. In this study, it was the first time to isolate CitCYP97A, CitCYP97B, and CitCYP97C from the citrus fruits. Phylogenetic analysis suggested that CitCYP97A was more closely related to CitCYP97C than to CitCYP97B. CitCYP97B contained three insertions in the amino acid sequence compared with CitCYP97A and CitCYP97C, and shared around 42% amino acid identity with CitCYP97A and CitCYP97C (Additional file 1: Figure S1). It has been reported that CYP97B3 in Arabidopsis was an uncharacterized cytochrome P450 monooxygenase, and the three amino acid insertions differentiated CYP97B from CYP97A and CYP97C [33, 45]. In addition, a transit peptide was predicted in the N-terminal reign of proteins encoded by CitHYb, CitCYP97A, CitCYP97B, and CitCYP97C, which suggested that the four carotene hydroxylases of citrus were able to import into plastids. As most carotenoids are synthesized and stored in plastids, the location of CitHYb, CitCYP97A, CitCYP97B, and CitCYP97C within plastid allows them to catalyze the reaction of carotene hydroxylation.

Fig. 6 HPLC analysis of carotenoids in α-carotene and β-carotene-accumulating E. coli BL21 (DE3) cells transformed with pRSF-2 Ek/LIC-CitHYb (b), pRSF-2 Ek/LIC-CitCYP97A (c), pRSF-2 Ek/LIC-CitCYP97B (d), and pRSF-2 Ek/LIC-CitCYP97C (e). Carotenoids extracted from the suspension cultures of α-carotene and β-carotene-accumulating E. coli BL21 (DE3) cells with pRSF-2 (empty vector) were used as control (a). β-Car, β-carotene; β-Cry, β-cryptoxanthin; Zea, zeaxanthin; Zein, zeinoxanthin.
Changes in carotenoid contents and expression of carotene hydroxylase genes in citrus fruits during the ripening process

In the previous studies, non-heme di-iron carotene hydroxylases were isolated from different plant species, and their roles in the carotenoid biosynthesis have been characterized [23–25, 46]. In Arabidopsis, BCH1 and BCH2 primarily catalyze the hydroxylation of the β-rings of β-carotene [22, 47]. Du et al. [48] reported that DSM2 gene (BCH) controlled the biosyntheses of zeaxanthin and ABA, and conferred drought and oxidative stress resistance in rice. In citrus fruits, massive accumulation of xanthophylls, especially ββ-xanthophylls, occurred during the ripening process. In the present study, the results showed that the expression of CitHYb increased gradually in the flavedo and juice sacs during the ripening process (Fig. 4). The increase in the expression of CitHYb was well in agreement with the accumulation of ββ-xanthophylls in the flavedo and juice sacs (Fig. 3). This result was consistent with the findings of Pons et al. [49], in which inhibiting the expression of CYP7-CHX (HYb) by RNA interference led to a significant increase of β-carotene (up to 36-fold) and a decrease of ββ-xanthophylls in sweet orange. Thus, it was suggested that CitHYb was a key gene for ββ-xanthophyll biosynthesis in citrus fruits.

In contrast to non-heme di-iron carotene hydroxylase, heme-containing cytochrome P450-type carotene hydroxylases preferentially hydroxylated the β- and α-rings of α-carotene, yielding lutein in Arabidopsis [30]. Quinlan et al. [31] found that a synergistic interaction existed between rice OsCYP97A4 and OsCYP97C2, which was required to drive the biosynthesis of lutein. In the present study, gene expression results showed that the expression of CitCYP97A and CitCYP97C increased with a peak in November in juice sacs, which might lead to an increase of lutein during the ripening process (Figs. 3c and 4b). In the flavedo, the expression of CitCYP97A decreased rapidly from August, which was in parallel with the reduction of lutein in the green stage. In the orange stage (from October), the expression of CitCYP97A and CitCYP97C increased with a peak in November, while the content of lutein decreased to a low level in the flavedo (Figs. 3b and 4a). In citrus fruits, a change from βα-carotenoid accumulation (α-carotene and lutein) to ββ-carotenoid accumulation (β-carotene, β-cryptoxanthin, zeaxanthin, all-trans-violaxanthin, and cis-violaxanthin) was observed in the flavedo during the ripening process, accompanying the disappearance of CitLCYe transcripts and the increase in CitLCYb transcripts. We previously reported that the expression of CitLCYe decreased rapidly to a low level in the orange stage in the flavedo of Satsuma mandarin [34, 39]. Thus, it was suggested that the level of lutein in the orange stage was mainly controlled by CitLCYe instead of CitCYP97A and CitCYP97C in the flavedo.

CYP97B is another member of CYP97 family with carotene hydroxylation activity. CYP97B3 of Arabidopsis exhibited a potential hydroxylation activity towards β-carotene and α-carotene [32, 33]. However, it remains controversial whether CYP97B is involved in the xanthophyll biosynthesis, because xanthophylls did not accumulate in the Arabidopsis quadruple mutant (bch1, bch2, cyp97c1, and cyp97a3) with only CYP97B3 [8, 24]. In the present study, the results showed that the expression level of CitCYP97B in the juice sacs was much lower than that of CitHYb, CitCYP97A, and CitCYP97C, indicating that CitCYP97B might not be a key gene for carotene hydroxylation in the juice sacs of citrus fruits (Fig. 4b).

Functional analysis of carotene hydroxylase genes in E. coli cells

In citrus, it is difficult to investigate the gene functions in the transgenic fruits because of its long juvenile phase that delays fruiting for 5–15 years [50]. As an alternative, E. coli cells that accumulate different carotenoids have been identified to be an efficient platform for investigating the functions of citrus carotenoid metabolic genes [11, 39, 43]. In the present study, we used the β-carotene-accumulating E. coli BL21 (DE3) cells and α-carotene- and β-carotene-accumulating E. coli BL21 (DE3) cells to investigate the functions of the four carotene hydroxylase genes of citrus. The results showed that CitHYb catalyzed the hydroxylation of the β-rings of β-carotene and α-carotene in E. coli BL21 (DE3) cells. When CitHYb was expressed in β-carotene-accumulating E. coli BL21 (DE3) cells, β-carotene was converted to β-cryptoxanthin and zeaxanthin, which supported the finding that CitHYb was a key gene for ββ-xanthophyll accumulation in citrus fruits (Fig. 5b). In Arabidopsis, it was reported that CYP97A3 and CYP97C1 were involved in lutein biosynthesis through hydroxylation of β- and ε-rings of α-carotene, respectively [24, 31]. However, in the absence of CYP97A the biosynthesis of lutein was not completely blocked. In Arabidopsis and rice, mutant of CYP97A only reduced around 20 % lutein compared with WT, which indicated that other carotene hydroxylases must also be able to catalyze hydroxylation of α-carotene on the β-ring [30, 51]. In the present study, we found that CitHYb participated in the biosynthesis of lutein. It converted α-carotene to zeinoxanthin, and the zeinoxanthin was further hydroxylated by CitCYP97C to produce lutein. Interestingly, CitCYP97C exhibited ε-ring hydroxylation activity only when it was co-expressed with CitHYb. Moreover, zeinoxanthin was the substrate for CitCYP97C instead of α-carotene, because α-cryptoxanthin, a monohydroxylated α-carotene on the ε-ring, was not detected in E. coli cells that transformed with CitCYP97C (Fig. 6e). These results suggested that α-carotene was hydroxylated on the β-ring and ε-ring sequentially to produce lutein in citrus.
fruits. A similar result was also reported in Arabidopsis, tomato and liverwort [29, 30, 52].

In contrast to the CitHYb, the carotene hydroxylation activities of CitCYP97A and CitCYP97B were not detected in E. coli BL21 (DE3) cells. In higher plants, only rice OsCYP97A4 was reported to exhibit β-ring hydroxylation activity in E. coli cells [31]. Amino acid sequence analysis showed that CitCYP97A shared 70 % similarity with OsCYP97A4, and the conserved oxygen-binding and heme-binding domains detected in CitCYP97A were identical to those of OsCYP97A4 (Additional file 2: Figure S2). In addition, we tested the activity of the full-length CitCYP97A, and optimized the culturing temperature and IPTG concentration as suggested in the study of Quinlan [45] (data not shown). Unfortunately, we could not detect any hydroxylation activity of CitCYP97A in E. coli cells. Similarly, attempts to assay the carotene hydroxylation activity of Arabidopsis CYP97A3 and liverwort MpCYP97A in E. coli cells were also failed [30, 52]. Whereas, mutant studies showed that Arabidopsis CYP97A3 (LU15 locus) exhibited a major hydroxylation activity towards the β-ring of α-carotene, and a minor activity towards the β-rings of β-carotene [30]. In orange carrot, a deficient CYP97A3 allele caused the accumulation of α-carotene and a high α/β-carotene ratio [53]. The research of Pons et al. [49] suggested that a second carotene hydroxylase might be present in sweet orange, because inhibiting the expression of Csβ-CHX (HYb) by RNA interference only caused a slight decrease in xanthophylls. In our present study, the gene expression results showed that CitCYP97A expressed in the flavedo and juice sacs during the ripening process. Meanwhile, the changes in CitCYP97A expression were consistent with the accumulation of lutein in the juice sacs (during the ripening stage) and flavedo (in the green stage), which indicating that CitCYP97A might be involved in the biosynthesis of lutein in citrus fruits. However, the mechanism that CitCYP97A regulates xanthophyll biosynthesis in citrus fruits seems to be more complicated, and some co-factors that are absent in E. coli cells might be needed for CitCYP97A to exert its activities. In the future research, further identification of the co-factors for heme-containing cytochrome P450-type carotene hydroxylases will contribute to elucidating the role of CitCYP97A in carotenoid accumulation.

Conclusion
In the present study, the roles of the four carotene hydroxylase genes (CitHYb, CitCYP97A, CitCYP97B, and CitCYP97C) in regulating xanthophyll biosynthesis were investigated in citrus fruits. The results showed that CitHYb was a key gene for β,β-xanthophyll biosynthesis in citrus fruits. Functional analysis showed that the CitHYb was able to hydroxylate the β-rings of β-carotene and α-carotene in E. coli BL21 (DE3) cells. Meanwhile, when CitHYb was co-expressed with CitCYP97C, α-carotene was hydroxylated on the β-ring and ε-ring sequentially to produce lutein. In addition, we detected the expression of CitCYP97A in citrus fruits during the ripening process, and the changes in its expression were consistent with the accumulation of lutein in the juice sacs (during the ripening stage) and flavedo (in the green stage), which indicating that CitCYP97A might be involved in the biosynthesis of lutein in citrus fruits. The results presented in this study will contribute to further elucidating the mechanism of carotenoid biosynthesis in citrus fruits, and provide new strategies to improve carotenoid composition of citrus fruits.

Methods
Plant material
Satsuma mandarin (Citrus unshiu Marc.) cultivated at the Fujieda Farm of Shizuoka University (Shizuoka, Japan) were used as materials. Fruit samples were collected periodically from August to December. The flavedo and juice sacs were separated from sampled fruits, immediately frozen in liquid nitrogen, and kept at −80 °C until used.

Extraction and determination of carotenoids
The identification and quantification of carotenoids were conducted according to the methods described by Ma et al. [11]. Pigments were extracted from the samples using a hexane:acetone (2:1 [v/v]) solution containing 0.1 % (w/v) 2,6-di-tert-butyl-4-methylphenol and 10 % (w/v) magnesium carbonate basic. After the organic solvents had been completely evaporated, the extracts containing carotenoids esterified to fatty acids were saponified with 20 % (w/v) methanolic KOH. Water-soluble extracts were then removed by adding NaCl-saturated water. The pigments repartitioned into the diethylether phase were recovered and evaporated to dryness. Subsequently, the residue was redissolved in 5 mL of a TBME: methanol (1:1 [v/v]) solution. An aliquot (20 μL) was separated by a reverse-phase HPLC system (Jasco, Tokyo, Japan) fitted with a YMC Carotenoid S-5 column of 250 × 4.6-mm-i.d. (Waters, Milford, MA) at a flow rate of 1 mL min⁻¹. The eluent was monitored by a photodiode array detector (MD-2015, Jasco). The carotenoid concentration was estimated by the standard curves and expressed as milligrams per gram fresh weight [34]. Carotenoid quantification was performed in three replicates.

Isolation and sequence analysis of carotene hydroxylase genes
Total RNA was extracted from the flavedo of Satsuma mandarin fruits according to the method described by Ikoma et al. [54]. First-strand cDNA was synthesized from 2 μg of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). The full-length cDNAs of
CitCYP97A, CitCYP97B, and CitCYP97C were amplified by RT-PCR using the primers designed within 5′ and 3′ UTRs according to the sequences obtained from the citrus genome database (Additional file 3: Table S1). The amplified cDNAs were cloned into the TOPO TA vector and 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).

The alignment of CitHyb, CitCYP97A, CitCYP97B, and CitCYP97C was created using CLUSTAL W (http://www.clustal.org). The Neighbor-joining phylogenetic tree was constructed based on the alignment of the deduced amino acid sequences of carotene hydroxylases using MEGA6 software [55]. Accession numbers are: Arabidopsis AtBCH1, AY113923; Arabidopsis AtBCH2, AY117225; Brassica napus BnBCH, EF026908; Coffea canephora CcBCH, DQ157165; Citrus CitHyb1, AB114653; Crocus sativus CsBCH2, AY579207; Lycopersicon esculentum SlCYP97-b1, Y14809; Lycopersicon esculentum SlCrt-b2, Y14810; Marchantia polymorpha MpBHY, AB981062; Narcissus tazetta var. chinensis NbBCH, JN625263; Zea mays ZmBCH1, GQ131287; Zea mays ZmHYD3, AY844958; Zea mays ZmHYD4, AY844956; Arabidopsis AtCYP97A3, NM_102914; Chlamydomonas reinhardtii CrCYP97A5, EF587911; Citrus CitCYP97A, LC143646; Daucus carota DcCYP97A3, JQ655297; Haematococcus pluvialis HpCYP97A, JX308236; Lycopersicon esculentum SlCYP97A29, EU849605; Lycium ruthenicum LrCYP97A, KF957714; Marchantia polymorpha McPcy97A, AB981063; Oryza sativa OsCYP79A4, AK068163; Vitis vinifera VvCYP79A, XP_002279984; Arabidopsis AtCYP97C1, AY424805; Citrus CitCYP97C, LC143648; Chlamydomonas reinhardtii CrCYP97C3, EF587910; Daucus carota DcCYP97C9, AB852076; Lycopersicon esculentum SlCYP97C11, EU849604; Medicago truncatula MtCYP97C10, ABC5 9096; Marchantia polymorpha McCYP79C, AB981065; Oryza sativa OsCYP97C2, AK065689; Arabidopsis AtCYP97B3, NM_117600; Citrus CitCYP97B, LC143647; Haematococcus pluvialis HpCYP97B, JX272918; Marchantia polymorpha McCYP97B, AB981064; Oryza sativa OsCYP97B2, XM_015771315; Ricinus communis RcCYP97B, XP_002520583; Sorghum bicolor ScCYP97B, XP_0022451628; Vitis vinifera VvCYP97B, XP_002266883. Predictions of transit peptides of CitHyb, CitCYP97A, CitCYP97B, and CitCYP97C were carried out using TargetP.

Total RNA extraction and real-time quantitative RT-PCR
Total RNA was extracted from the flavedo and juice sacs of Satsuma mandarin at different stages according to the method described by Ikoma et al. [54]. The total RNA was cleaned up using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion. The reverse transcription (RT) reaction was 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 CitHyb, CitCYP97A, CitCYP97B, and CitCYP97C were designed with the Primer Express software (Additional file 4: Table S2). 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) 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.

Functional analysis of the carotene hydroxylases in E. coli cells
The cDNAs of CitHyb, CitCYP97A, CitCYP97B, and CitCYP97C without transit peptide were cloned into the pRSF-2 Ek/LIC vector or pCDF-2 Ek/LIC vector, respectively. In the previous study, two recombinant plasmids pET-CitLCYb1 and pET-CitLCYe were constructed, and transformed into E. coli BL21 (DE3) cells harboring a lycopene biosynthetic plasmid pACCRT-MIB, respectively [39, 56]. The co-transformation of pACCRT-MIB with pET-CitLCYb1 or pET-CitLCYe led to β-carotene accumulation or α-carotene and β-carotene-accumulation in the E. coli BL21 (DE3) cells. In the present study, the recombinant plasmids pRSF-2-CitHyb, pRSF-2-CitCYP97A, pRSF-2-CitCYP97B, and pRSF-2-CitCYP97C were transformed into the β-carotene-accumulating, as well as α-carotene and β-carotene-accumulating E. coli BL21 (DE3) cells. To investigate the interactions among the carotene hydroxylases of citrus, we co-expressed pCDFS-2-CitCYP97C with pRSF-2-CitHyb, pRSF-2-CitCYP97A, and pRSF-2-CitCYP97B in the α-carotene and β-carotene-accumulating E. coli BL21 (DE3) cells, respectively. After induction with 0.05 M isopropyl β-D-thiogalactoside (IPTG) for 2 d at 27 °C, carotenoids were extracted from E. coli cells. 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 the HPLC analysis. The freeze-ground material was extracted with a mixture of chloroform and methanol (2:1 [v/v]) until all the color was removed from the E. coli cells. The carotenoid extracts
were reduced to dryness by rotary evaporation, and then dissolved in the methyl tert-butyl ether: methanol (1:1 [v/v]) solution containing 0.1 % butylated hydroxytoluene. α-Carotene, β-carotene, β-cryptoxanthin, zeaxanthin, and lutein were identified by comparing their specific retention times and absorption spectra with the authentic standards (Kato et al. 2004). The identification of zeinoxanthin was conducted using the methods described by Meléndez-Martínez et al. [57]. For each carotene hydroxylase gene, three replicates were conducted using different colonies in the functional analysis in *E. coli* cells.

### Statistical analysis

All values are shown as the mean ± SE for three replicates. The data were analyzed, and Tukey’s HSD test (at *P* < 0.05) was used to compare the treatment means.

### Additional files

- **Additional file 1:** Figure S1. Three sequence insertions in CitCYP97B compared with that of CitCYP97A and CitCYP97C. (DOCX 38 kb)
- **Additional file 2:** Figure S2. Alignment of deduced amino acid sequences of CitCYP97A and OsCYP97A4. The alignment was created using CLUSTAL W ([http://www.clustal.org)](http://www.clustal.org). (DOCX 153 kb)
- **Additional file 3:** Table S1. Primer sequences used for isolating full-length cDNAs of CitCYP97A, CitCYP97B, CitCYP97C. (DOCX 12 kb)
- **Additional file 4:** Table S2. Probes used for the quantitative RT-PCRs of carotene hydroxylase genes. (DOCX 38 kb)

### Abbreviations

- CYP, cytochrome P450
- HYb, β-ring hydroxylase
- LCYb, lycopene β-cyclase
- LCYe, lycopene e-cyclase
- ZEP, zeaxanthin epoxidase

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### Availability of data and materials

The data sets supporting the results of this article are included within the article and its additional files.

### Authors’ contributions

GM and MK conceived and designed the study, WY and IT conducted the experiments and collected the data, NI and MO analyzed the data, KY and MV provided plant materials and reagents, LZ and MK prepared the manuscript. All authors read and approved the manuscript.

### Completing interests

The authors declare that they have no completing interests.

### Consent for publication

Not applicable.

### Ethics approval and consent to participate

Not applicable.

### References

1. Cunningham FX, Gantt E. Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol. 1998;49:557–83.
2. Havaux M. Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci. 1998;3:147–51.
3. Cazzonelli CI, Pogson BJ. Source to sink: regulation of carotenoid biosynthesis in plants. Trends Plant Sci. 2010;15:266–74.
4. Nisar N, Li L, Lu S, Khin NC, Pogson BJ. Carotenoid Metabolism in Plants. Mol Plant. 2015;8:68–82.
5. Niyogi KK, Björkman O, Grossman AR. The roles of specific xanthophylls in photoprotection. Proc Natl Acad Sci U S A. 1997;94:14162–7.
6. Croce R, Weiss S, Bassi R. Carotenoid-binding sites of the major light-harvesting complex II of higher plants. J Biol Chem. 1999;274:20613–23.
7. Liu Z, Yan H, Wang K, Kuan T, Zhang J, Gui L, et al. Crystal structure of spinach major light-harvesting complex at 2.7 Å resolution. Nature. 2004;428:287–92.
8. Fiore A, Dell’Osto L, Cazzaniga S, Dietero G, Giuliano G, Bassi R. A quadruple mutant of Arabidopsis displays a β-carotene hydroxylation activity for LUT/1 CYP97C1 and a regulatory role of xanthophylls on determination of the P32/PS2 ratio. BMC Plant Biol. 2012;12:52.
9. Kato M, Matsumoto H, Ikoma Y, Okuda H, Yano M. The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. J Exp Bot. 2006;57:2153–64.
10. Walter MH, Floss DS, Stack D. Apocarotenoids: hormones, mycorrhizal metabolites and aroma volatiles. Planta. 2010;232:1–17.
11. Ma G, Zhang LC, Matsuura A, Matsuani K, Yamawaki K, Yahata M, et al. Enzymatic formation of β-citraurin from β-cryptoxanthin and zeaxanthin by carotenoid cleavage dioxygenase4 in the flavedo of citrus fruit. Plant Physiol. 2013;163:682–95.
12. Rodrigo MJ, Alquézar B, Alós E, Lado J, Zacarías L. Biochemical bases and molecular regulation of pigmentation in the peel of Citrus fruit. Sci Hortic. 2013;163:46–52.
13. Ahaszem O, Rubio-Moraga A, Berman J, Capell T, Christou P, Zhu C, Gómez-Gómez L. The carotenoid cleavage dioxygenase CDC2 catalysing the synthesis of crocetin in spring crocuses and saffron is a plastidial enzyme. New Phytol. 2016;209:650–63.
14. Cerhan JR, Saag KG, Merlino LA, Mirkus TR, Criswell LA. Antioxidant micronutrients and risk of rheumatoid arthritis in a cohort of older women. Arthritis Rheum. 2003;47:7345–54.
15. Khachik F, London E, de Moura FF, Johnson M, Steidl S, Detolla L, et al. Chronic ingestion of (3R,3′R)–lutein and (3R,3′R)–zeaxanthin in the female rhesus macaque. Invest Ophthalmol Vis Sci. 2006;47:5476–86.
16. Yamaguchi M, Hamamoto R, Uchiyama S, Ishiyama K, Hashimoto K. Anabolic effects of bee pollen cistus ladaniferus extract on bone components in the femoral-diaphyseal and metaphyseal tissues of rats in vitro and in vivo. J Health Sci. 2006;52:43–9.
17. Sugiuira M, Nakamura M, Oqawa K, Ikoma Y, Ando F, Shimokata H, Yano M. Dietary patterns of antioxidant vitamin and carotenoid intake associated with bone mineral density: findings from post-menopausal Japanese female subjects. Osteoporos Int. 2011;22:143–52.
18. Takayanagi K, Morimoto S, Shirakura Y, Mukai K, Sugiyama T, Tokuji Y, Ohnishi M. Mechanism of visceral fat reduction in Tsumura Suzuki obese, diabetes mellitus (TSOD) mice orally administered (3R,3′R)–cryptoxanthin from Satsuma mandarin oranges (Citrus unshiu Marc). J Agric Food Chem. 2011;59:12342–51.
19. Yamaguchi M. Role of carotenoid β-cryptoxanthin in bone homeostasis. J Biomed Sci. 2012;19:36.
20. Iskandar AR, Liu C, Smith DF, Hu KQ, Choi SW, Ausman LM, Wang XD. β-Cryptoxanthin restores nicotine-reduced lung SIRT1 to normal levels and inhibits nicotine-promoted lung tumorigenesis and emphysema in A/J mice. Cancer Prev Res (Phila). 2013;6:309–20.
21. Poucché C, Galan P, Ducros Y, Latino-Martel P, Herberg S, Touvier M. Plasma carotenoids and retinol and overall and breast cancer risk: a nested case–control study. Nutr Cancer. 2014;66:980–8.
22. Tian L, DellaPenna D. Characterization of a second carotenoid beta-hydroxylase gene from Arabidopsis and its relationship to the LUT1 locus. Plant Mol Biol. 2001;47:379–88.

23. Galpaz N, Ronen G, Khafaa Z, Zamer D, Hirscheberg J. A chloroplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato white-flower locus. Plant Cell. 2006;18:1947–60.

24. Kim J, Smith JJ, Tian L, DellaPenna D. The evolution and function of carotenoid hydroxylases in Arabidopsis. Plant Cell Physiol. 2009;50:463–79.

25. D’Ambrosio C, Stigliani AL, Gorio G. Overexpression of CrtB2 (carotene beta-hydroxylase 2) from S. lycopersicum L. differentially affects xanthophyll synthesis and accumulation in transgenic tomato plants. Transgenic Res. 2011;20:47–60.

26. Tian L, Magallanes-Lundback M, Musetti V, DellaPenna D. Functional analysis of beta- and epsilon-ring carotenoid hydroxylases in Arabidopsis. Plant Cell. 2003;15:1320–32.

27. Tian L, Musetti V, Kim J, Magallanes-Lundback M, DellaPenna D. The Arabidopsis LUT1 locus encodes a member of the cytochrome p450 family that is required for carotenoid epsilon-ring hydroxylation activity. Proc Natl Acad Sci U S A. 2004;101:402–7.

28. Fiore A, Dall’Osto L, Fraser PD, Bassi R, Giuliano G. Elucidation of the beta-carotene hydroxylation pathway in Arabidopsis thaliana. FEBS Lett. 2006;580:4718–22.

29. Stigliani AL, Gorio G, D’Ambrosio C. Characterization of P450 carotenoid beta- and epsilon-hydroxylases of tomato and transcriptional regulation of xanthophyll biosynthesis in root, leaf, petal and fruit. Plant Cell Physiol. 2011;52:851–65.

30. Kim J, DellaPenna D. Defining the primary route for lutein synthesis in plants: the role of Arabidopsis carotenoid beta-ring hydroxylase CYP97A3. Proc Natl Acad Sci U S A. 2006;103:3474–9.

31. Quinlan RF, Shumskaya M, Bradbury LM, Beltrán J, Ma C, Kennelly EJ, Wurtzel ET. Synergistic interactions between carotenoid ring hydroxylases drive lutein formation in plant carotenoid biosynthesis. Plant Physiol. 2012;160:204–14.

32. Kim JE, Punja ZK, Douglas CJ. Co-expression of Arabidopsis thaliana cytochrome P450 enzymes and NADPH-cytochrome P450 reductase in Escherichia coli col: testing the function of candidate beta-carotene hydroxylases. In: P450 Systems and Regulation, Proceedings of 14th International Conference on Cytochromes P450, Dallas, TX. Italy: Medimond S.r.l; 2005. p. 115–20.

33. Kim JE, Cheng KM, Craft NE, Hamberger B, Douglas CJ. Over-expression of Arabidopsis thaliana carotenoid hydroxylases individually and in combination with a beta-carotene ketolase provides insight into in vivo functions. Phytochemistry. 2010;71:168–78.

34. Kato M, Ikoma Y, Matsumoto H, Sugiyama M, Hyodo H, Yano M. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiol. 2004;134:2824–37.

35. Rodrigo MJ, Marcos JF, Zacarías L. Biochemical and molecular analysis of carotenoid biosynthesis in flowered orange (Citrus sinensis L.) during fruit development and maturation. J Agric Food Chem. 2004;52:6724–31.

36. Rodrigo MJ, Zacarías L. Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic genes in the flowered orange (Citrus sinensis L. Osbeck). Postharvest Biol Technol. 2007;48:14–22.

37. Rios G, Naranjo MA, Rodrigo MJ, Alos E, Zacarías L, Cerós M, Talón M. Identification of a GCC transcription factor responding to fruit colour change events in citrus through the transcriptomic analyses of two mutants. BMC Plant Biol. 2010;10:276.

38. Wei X, Chen C, Yu Q, Gao A, Yu Y, Liang G, Gmitter FG Jr. Comparison of carotenoid accumulation and biosynthetic gene expression between Valencia and Rohde Red Valencia sweet oranges. Plant Sci. 2014;222:278–28.

39. Zhang L, Ma G, Shira Y, Kato M, Yamawaki K, Ikoma Y, Matsumoto H. Expression and functional analysis of two lycopene beta-cyclases from citrus fruits. Planta. 2011;236:3135–25.

40. Bouvier F, Keller Y, d’Harlingue A, Camara B. Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (Capsicum annuum L.). Biochim Biophys Acta. 1998; 1391:320–8.

41. Diietro G, Welsch R, Tavazza R, Mourgues F, Pizizichini D, Beyer P, Giuliano G. Silencing of beta-carotene hydroxylase increases total carotenoid and beta-carotene levels in potato tubers. BMC Plant Biol. 2007;7:11.

42. Li Q, Farre G, Naqvi S, Breitenbach J, Sanahuja G, Bai C, Sandmann G, Capell T, Christou P, Zhu C. Cloning and functional characterization of the maize carotenoid isomerase and beta-carotene hydroxylase genes and their regulation during endosperm maturation. Transgenic Res. 2010;19:1033–68.

43. Alquézar B, Zacarías L, Rodríguez MJ. Molecular and functional characterization of a novel chloroplast-specific lycopene beta-cyclase from citrus and its relation to lycopene accumulation. J Exp Bot. 2009;60:1783–97.

44. Zhang L, Ma G, Kato M, Yamawaki K, Takagi T, Kiriya Y, Ikoma Y, Matsumoto H, Yoshioka T, Nesumi H. Regulation of carotenoid accumulation and the expression of carotenoid metabolic genes in citrus juice sacs in vitro. J Exp Bot. 2012;63:871–86.

45. Quinlan RF, Jaradat TT, Wurtzel ET. Escherichia coli as a platform for functional expression of plant P450 carotenoid hydroxylases. Arch Biochem Biophys. 2007;458:146–57.

46. Wang HM, To KY, Lam MJ. Modification of flower colour by suppressing beta-carotene hydroxylases in Oncidium. Plant Biol (Stuttg). 2016;18:220–9.

47. Sun Z, Gant E, Cunningham Jr FX. Cloning and functional analysis of the beta-carotene hydroxylase of Arabidopsis thaliana. J Biol Chem. 1996;271:24349–52.

48. Du H, Wang N, Cui F, Li X, Xiao J, Xiong L. Characterization of the beta-carotene hydroxylase gene DSM2 conferring drought and oxidative stress resistance by increasing xanthophyll and abscisic acid synthesis in rice. Plant Physiol. 2010;154:1304–18.

49. Pons E, Alquézar B, Rodríguez A, Martorell P, Genovés S, Ramón D, Rodrigo MJ, Zacarías L, Peña L. Metabolic engineering of beta-carotene in orange fruit increases its in vivo antioxidant properties. Plant Biotechnol J. 2014;12:17–27.

50. Peña L, Cervera M, Fagoaga C, Romero J, Ballester A, Soler N, Pons E, Rodríguez A, Peris J, Juarés J, Navarro L, Citrus. In C. Kole, TC. Hall. Citrus. In: Kole C, Hall TC, editors. Compendium of Transgenic Crop Plants: Tropical and Subtropical Fruits and Nuts. Oxford, UK: Blackwell Publishing. 2008. p. 1–62.

51. Liu MZ, Zhao DY, Shan JX, Zhu MZ, Shi M, Gao JP, Lin HK. Rice carotenoid beta-carotene hydroxylase CYP97A4 is involved in lutein biosynthesis. Plant Cell Physiol. 2012;53:987–1002.

52. Takemura M, Maoka T, Misawa N. Biosynthetic routes of hydroxylated carotenoids (xanthophylls) in Marchantia polymorpha, and production of novel and rare xanthophylls through pathway engineering in Escherichia coli. Plant. 2015;241:699–710.

53. Arango J, Jourdan M, Geoffrèau E, Beyer P, Welsh R. Carotenoid hydroxylase activity determines the levels of both a-carotene and total carotenoids in orange carrots. Plant Cell. 2014;26:2223–33.

54. Ikoma Y, Yano M, Ogawa K, Yoshioka T, Xu ZC, Hisada S, Omura M, Moriguchi T. Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit for quality by cDNA library construction and RT-PCR. J Jpn Soc Hortic Sci. 1996;64:809–14.

55. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.

56. Misawa N, Shimada H. Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. Journal of Biotechnology. 1998;59:169–81.

57. Meléndez-Martínez AJ, Britton G, Vicario IM, Heredia FJ. Identification of zeaxanthin in orange juices. J Agric Food Chem. 2005;53:6362–7.