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E-mail: jzus_b@zju.edu.cn

Correspondence

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Carotenoid components and their biosynthesis in a bud mutant of Shiranui mandarin (*Citrus reticulata* Blanco) with citrine flavedo

Xun WANG[1*], Jinqiu HUANG[2*], Zongyan YIN[2], Ke XU[3], Dong JIANG[4], Lijin LIN[1], Xiaoai ZHANG[2], Zhihui WANG[2✉]

[1]*Institute of Pomology and Olericulture, Sichuan Agricultural University, Chengdu 611130, China*
[2]*College of Horticulture, Sichuan Agricultural University, Chengdu 611130, China*
[3]*Sichuan Horticulture Techniques Promotion Station, Chengdu 610041, China*
[4]*Citrus Research Institute, Southwest University, Chongqing 400712, China*

Carotenoids are secondary metabolite responsible for colored pigments in plants and microbes (Li et al., 2022). They are a class of C_{40} tetraterpenoids consisting of eight isoprenoid units, and can be classified into carotenes and xanthophylls on the basis of their functional groups (Saini et al., 2015). Carotenes can be linear (phytoene, phytofluene, and ζ-carotene) or branched (β-carotene and α-carotene). Xanthophylls comprise β,β-xanthophylls (β-cryptoxanthin, zeaxanthin, violaxanthins, and neoxanthin) and β,ε-xanthophylls (α-cryptoxanthin, α-carotene, and lutein). Citrus fruits are complex sources of carotenoids, which are the principal pigments responsible for the typical orange color of most types (Chen, 2020). The difference in total carotenoid content and the diversity of carotenoid isomer proportion also accounts for other colors of citrus fruits, such as yellow, red, and pink (Chen, 2020).

Carotenoid biosynthesis in higher plants is initiated by the linkage of two C_{20} precursors (Hirschberg, 2001). More than nine catalases participate in this pathway, including phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), those responsible for β,β-carotenoids such as lycopene β-cyclase (LCYb), β-carotene hydroxylase (HYb), zeaxanthin epoxidase (ZEP), and neoxanthin synthase (NSY), and those responsible for β,ε-carotenoids such as LCYb, lycopene ε-cyclase (LCYe), and ε/β-carotene hydroxylase (HYe and HYb). The accumulation of carotenoid components is usually coincident with the difference in catalase gene transcript levels in the biosynthetic pathway. Occasionally, there is a feedback regulatory mechanism resulting in inconsistency between the transcription of carotenoid genes and the accumulation of carotenoid components (Feng et al., 2021).

Citrine Shiranui (also named "Jinlegan") is a spontaneous bud mutation of Shiranui mandarin (*Citrus reticulata* Blanco) found in Southwest China. Its most striking feature is the brighter colored (citrine/yellow) flavedo of mature fruit, whereas the normal flavedo of Shiranui is a rich orange color (Fig. 1a). In this research, the differences in carotenoid biochemistry and biosynthesis of Citrine Shiranui and Shiranui were analyzed to illuminate the phenotype of this citrus mutation. We analyzed the total carotenoid content, carotenoid component proportions (using high-performance liquid chromatography (HPLC) analysis), and transcript levels of carotenoid biosynthesis genes (using quantitative real-time polymerase chain reaction (qRT-PCR)) in the two cultivars at six stages of fruit ripening, including breaker stage (Stage I), coloring stage (Stage II), full coloring stage (Stage III), one month after full coloring (Stage IV), two months after full coloring (Stage V), and at the full ripening stage (Stage VI). The detailed methods are included in the supplementary.

The total carotenoid contents were 4.23–7.33 times higher in Shiranui than in Citrine Shiranui at all stages of fruit ripening (Fig. 1b), which was considered to be the primary cause of the difference in flavedo color. The same mechanism generally occurs in flesh or flavedo pigmentation mutants of citrus.

✉ Zhihui WANG, wangzhihui318@126.com
[*] The two authors contributed equally to this work
Zhihui WANG, https://orcid.org/0000-0003-2234-144X

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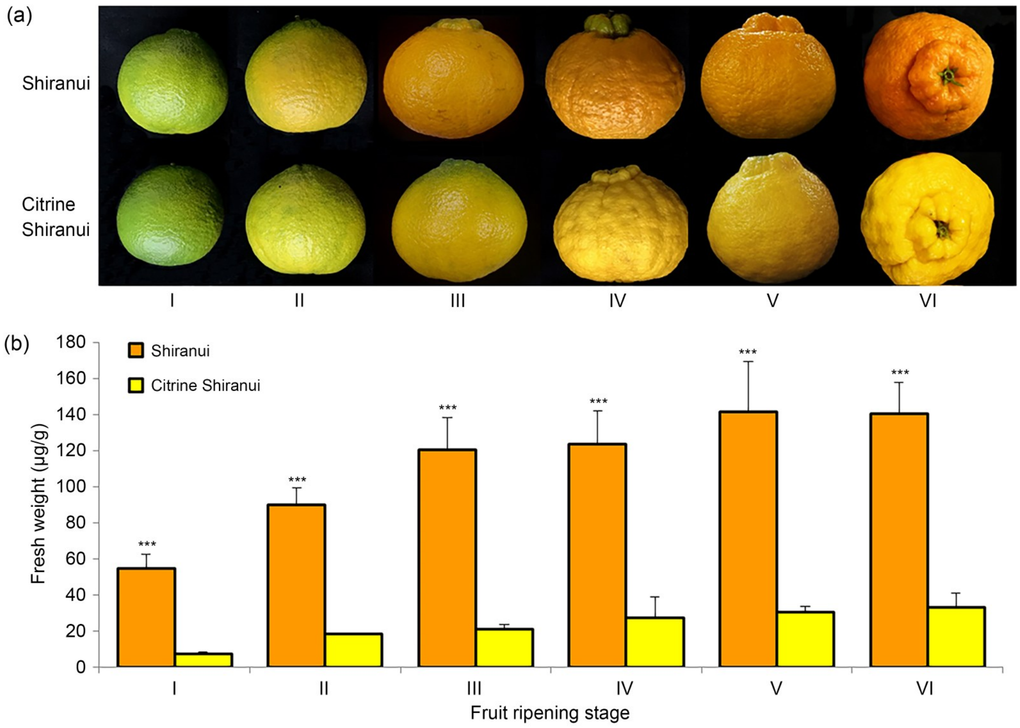

Fig. 1 Appearance of flavedos and total carotenoid contents. (a) The appearance of flavedos of Shiranui and Citrine Shiranui at six stages of fruit ripening. (b) The total carotenoid contents. Data were expressed as mean±standard deviation (SD), *n*=3. A significance test was conducted: *** *P*<0.001 vs. Citrine Shiranui. I: breaker stage; II: coloring stage; III: full coloring stage; IV: one month after full coloring; V: two months after full coloring; VI: the full ripening stage.

In the red-pigmented sweet orange mutant Cara Cara (*Citrus. sinensis* Osbeck), the total carotenoid is increasingly accumulated compared with the yellowish-orange color of the parent Navel orange flavedo (Alquezar et al., 2008). The sweet orange Pinalate is a mutant of Navelate and produces distinctive yellow fruit instead of the typical bright orange fruit of Navelate. In full-colored Pinalate fruit, the total carotenoid content is significantly lower than that in Navelate fruit (Rodrigo et al., 2003). The yellow flavedo of lemon (*Citrus. limon* (L.) Osbeck) has a low total carotenoid content compared with the orange flavedo of Satsuma mandarin (*Citrus. unshiu* Marc.) and Valencia sweet orange (Kato et al., 2004).

All of twelve prevalent components of the carotenoid biosynthesis pathway were identified in the flavedo of Shiranui and Citrine Shiranui (Fig. 2). Five carotenoids, namely 9-*cis*-violaxanthin, *trans*-violaxanthin, β-cryptoxanthin, phytoene, and α-cryptoxanthin, were increased in proportion with the fruit coloring and ripening processes in both cultivars (Fig. 3). Conversely, β-carotene, neoxanthin, α-carotene, and lutein were the major components at the breaker stage (Stage I) in both cultivars, but they were decreased in proportion with the coloring and ripening of the fruit (Fig. 3). Notably, the proportions of lutein and neoxanthin decreased sharply in Shiranui (both with *P*<0.001), but to a lower degree in Citrine Shiranui.

Violaxanthins (including 9-*cis*-violaxanthin and *trans*-violaxanthin) are the carotenoids mainly accumulated in the flavedo of mature fruit in various citrus with an orange color, especially in mandarin (Kato et al., 2004; Agócs et al., 2007). In the present research, violaxanthins were the principal conventional carotenoids in both Shiranui and Citrine Shiranui (Fig. 3) from the full coloring stage (Stage III) to the full ripening stage (Stage VI).

In Shiranui, β-cryptoxanthin was another predominant carotenoid after violaxanthins (including 9-*cis*-violaxanthin and *trans*-violaxanthin), whereas it was less accumulated in Citrine Shiranui (Fig. 3). β-Cryptoxanthin is mainly accumulated in the flavedo of mature fruit in various mandarin citruses with an orange color, but is present at a low concentration in green-yellow organs, such as immature fruits and

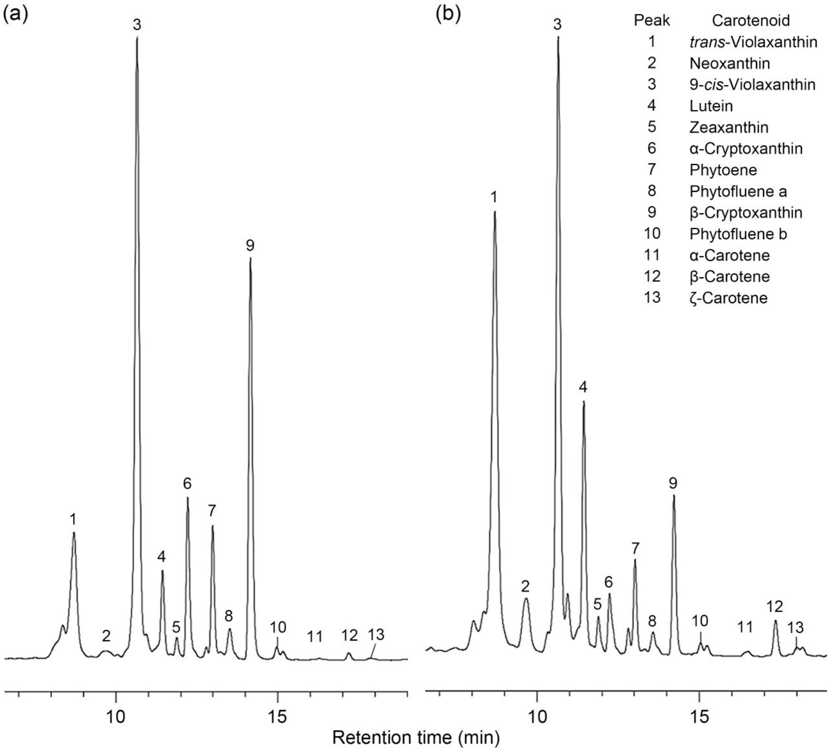

Fig. 2 High-performance liquid chromatography analysis (HPLC) chromatogram of carotenoids in the flavedos of Shiranui (a) and Citrine Shiranui (b) at the full ripening stage (Stage VI).

leaves (Sumiasih et al., 2017). Consequently, the lower accumulation of β-cryptoxanthin in the Citrine Shiranui flavedo was a possible reason for its off-orange color.

A more notable difference in the two citrus cultivars was observed in the accumulation pattern of lutein and neoxanthin. These carotenoids were present in the flavedo at relatively high proportions at the beginning of the color transition (break stage/Stage I) and decreased as the fruit ripened in the both two citrus cultivars (Fig. 3). However, the transition in Citrine Shiranui seemed incomplete, resulting in the retention of greater quantities of lutein and neoxanthin compared to Shiranui (Fig. 3). In particular, lutein in Citrine Shiranui was the second most abundant carotenoid after violaxanthins during the period after color transition. Generally, lutein and neoxanthin are the preponderant carotenoid components in immature fruits and leaves (Rodrigo et al., 2003), and they are derivatively strongly associated with their green-yellow color. Therefore, the retention of lutein and neoxanthin contributed to the yellow flavedo of Citrine Shiranui.

The analysis of the carotenoid components of so-called "colorless" citrus cultivars with a bright yellow flavedo indicated the excessive accumulation of colorless compounds (phytoene, phytofluene, or ζ-carotene), such as in lemon (Chen, 2020), yellow-pigmented sweet orange Pinalate (Rodrigo et al., 2003), and white grapefruit (*Citrus paradisi* Macf.) (Alquezar et al., 2013). In the present research on Citrine Shiranui, some differences from the results of previous research were noted. Less β-cryptoxanthin (responsible for an orange color), coupled with higher proportions of lutein and neoxanthin (associated with a green-yellow color), was a considerable factor underlying these deviations.

The expression of the above nine carotenoid biosynthesis-related genes was investigated. All of them showed an initial increase and subsequent decrease in transcript level during fruit ripening in both Shiranui and Citrine Shiranui (Fig. 4). Comparing the two cultivars, all genes were generally more highly expressed in Shiranui than in Citrine Shiranui, especially during the initial three stages (Fig. 4), which might explain the greater accumulation of total carotenoids and most carotenoid components in the Shiranui fruit flavedo. Especially, higher transcript levels of *PSY* may provide abundant substrate for the subsequent steps and sustain a higher total carotenoid content in

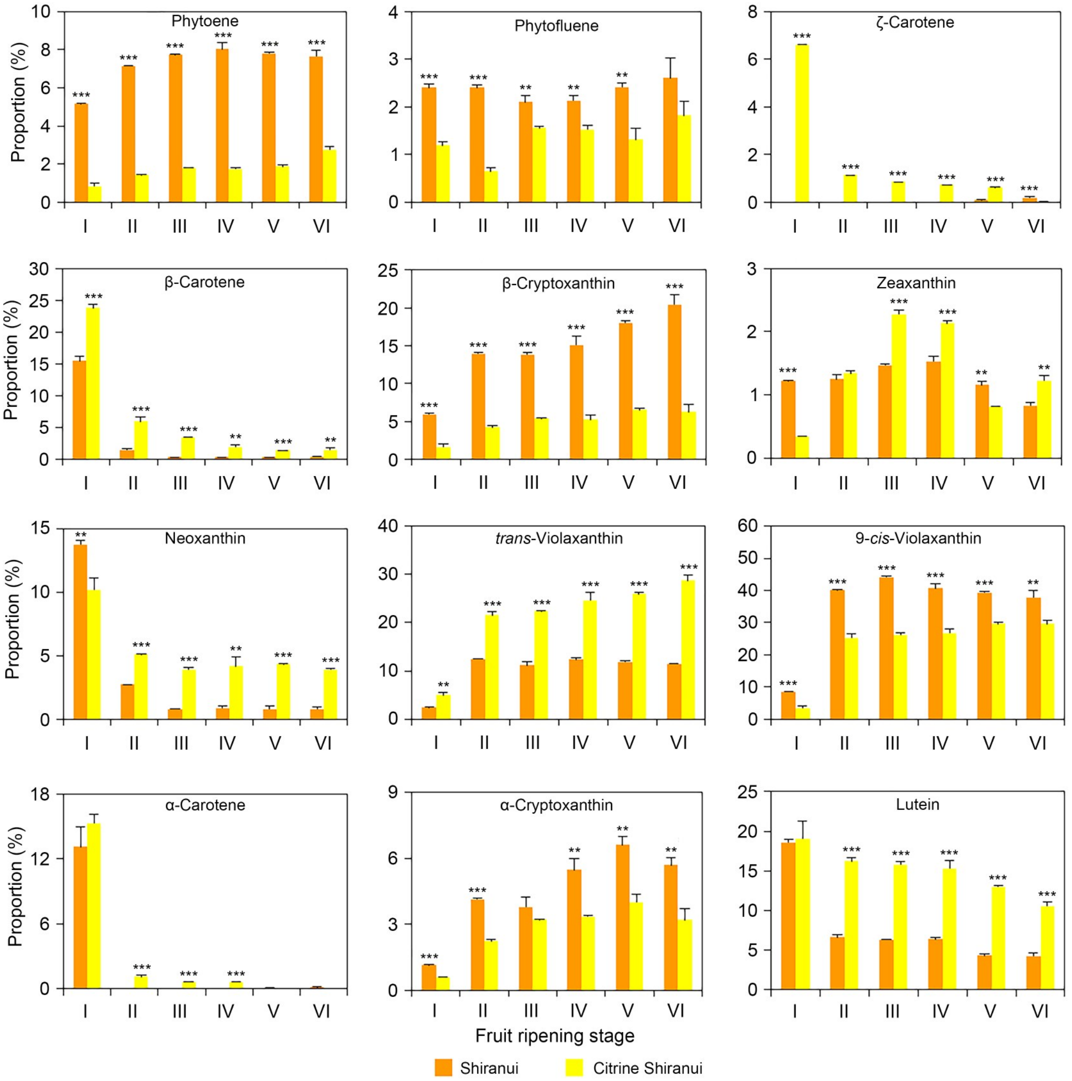

Fig. 3 Carotenoid proportions (%) in flavedos. Data were expressed as mean±standard deviation (SD), *n*=3. A significance test was conducted: * *P*<0.001, ** *P*<0.01 vs. Citrine Shiranui. I: breaker stage; II: coloring stage; III: full coloring stage; IV: one month after full coloring; V: two months after full coloring; VI: the full ripening stage.**

the flavedo of Shiranui, since PSY catalyzes a rate-limiting step in carotenoid biosynthesis (Rodríguez-Villalón et al., 2009).

In each seperated cultivar, the relative expression levels of all nine carotenoid genes during all stages were calculated (Fig. 5). The relative transcript level of *HYb* was higher than that of other carotenoid biosynthetic genes in Shiranui and Citrine Shiranui (Fig. 5). This HYb is the isogene of β-carotene hydroxylase 2 (BCH2/At5g52570) of *Arabidopsis*, which mainly catalyzes the hydroxylation of β-carotene and β-cryptoxanthin, and partially participates in α-carotene hydroxylation (Ruiz-Sola and Rodríguez-Concepción, 2012). The high transcript level of *HYb* here might accord with its multiple roles. A similar phenomenon was observed by several scholars, such as *CHYB2* in watermelon (*Citrullus lanatus*) (Yuan et al., 2021) and *Ag10G02588.1* in celery (*Apium graveolens*) (Song et al., 2022). Moreover, since the differential expression of *HYb*/*BCH* genes provides flexibility to regulate the accumulation of downstream xanthophylls or upstream carotenes (Ruiz-Sola and Rodríguez-Concepción, 2012), the accumulation of violaxanthins in the present study might be predicted from the high transcript level of *HYb*.

The *ZEP* transcript level was lower than the upstream *HYb* and downstream *NSY* for both cultivars (Fig. 5), although the product of ZEP protain (violaxanthins) was massively accumulated. Regulations of

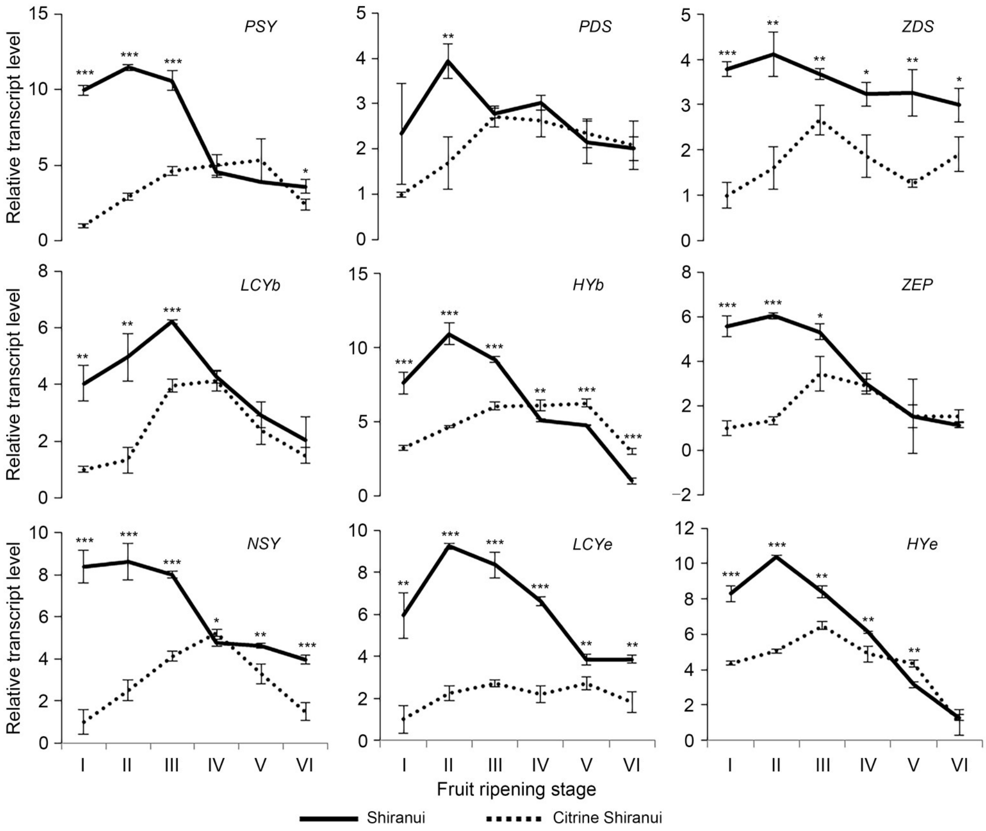

Fig. 4 Expression of carotenoid biosynthesis genes (the value was normalized to the minimum among all the messenger RNA (mRNA) levels in both two cultivars for each gene). Data were expressed as mean±standard deviation (SD), *n*=3. A significance test was conducted: *** *P*<0.001, ** *P*<0.01, and * *P*<0.05 vs. Citrine Shiranui. *PSY*: phytoene synthase; *PDS*: phytoene desaturase; *ZDS*: ζ-carotene desaturase; *LCYb*: lycopene β-cyclase; *HYb*: β-carotene hydroxylase; *ZEP*: zeaxanthin epoxidase; *NSY*: neoxanthin synthase; *LCYe*: lycopene ε-cyclase; *HYe*: ε-carotene hydroxylase; I: breaker stage; II: coloring stage; III: full coloring stage; IV: one month after full coloring; V: two months after full coloring; VI: the full ripening stage.

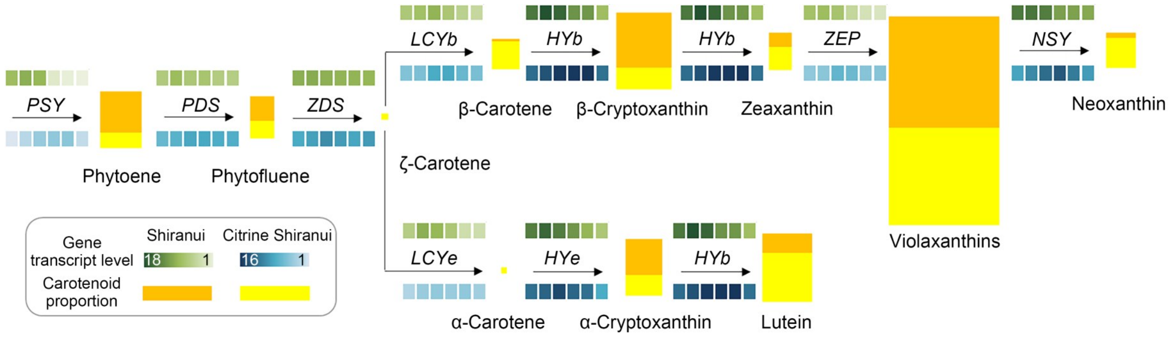

Fig. 5 Schematic diagram of carotenoid biosynthetic pathways. The value of carotenoid biosynthesis genes was normalized to the minimum among all messenger RNA (mRNA) levels in all genes for either Shiranui or Citrine Shiranui. The square area indicates the proportion of carotenoid component at the full ripening stage (Stage VI). *PSY*: phytoene synthase; *PDS*: phytoene desaturase; *ZDS*: ζ-carotene desaturase; *LCYb*: lycopene β-cyclase; *HYb*: β-carotene hydroxylase; *ZEP*: zeaxanthin epoxidase; *NSY*: neoxanthin synthase; *LCYe*: lycopene ε-cyclase; *HYe*: ε-carotene hydroxylase.

carotenoid accumulation at multiple levels, such as feedback regulation by end products, were frequently detected (Stanley and Yuan, 2019). The finding of the present research might implicate a potential negative feedback regulation in *ZEP* transcription, i.e., the accumulation of violaxanthins or downstream carotenoids/products might lead to the downregulation of *ZEP*. Further study is needed to confirm this notion.

The analyses of this paper provide new insights into the carotenoid biochemistry and biosynthesis of the mutant citrus with brighter color flavedo. Lower β-cryptoxanthin (a carotenoid associated with an orange color) combined with higher proportions of lutein and neoxanthin (associated with a green-yellow color) were found as additional factors contributing to the brighter color flavedo of Citrine Shiranui. The significantly higher transcript levels of *PSY* in the early stages of fruit ripening in the flavedo of Shiranui might provide abundant substrate to sustain its higher total carotenoid content. The *HYb* gene was more highly expressed than the other genes in both cultivars, which is in accord with its multiple hydroxylation functions. Meanwhile, the low transcript levels of *ZEP* probably imply potential negative feedback regulation through violaxanthins or downstream carotenoids/metabolic products.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Xun WANG, Jinqiu HUANG, and Zongyan YIN performed the experimental research and data analysis. Ke XU contributed to the study design. Dong JIANG provided the information about the plant materials. Xun WANG, Lijin LIN, and Xiaoai ZHANG contributed to data analysis, writing and editing of the manuscript. Zhihui WANG financially supported this work. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Xun WANG, Jinqiu HUANG, Zongyan YIN, Ke XU, Dong JIANG, Lijin LIN, Xiaoai ZHANG, and Zhihui WANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Materials and methods