Identifying a Carotenoid Cleavage Dioxygenase (CCD4) Gene Controlling Yellow/White Fruit Flesh Color of “Piqiutao” (White Fruit Flesh) and Its Mutant (Yellow Fruit Flesh)

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Abstract

To better understand the fruit flesh coloration mechanism of peach (Prunus persica), the composition and accumulation of carotenoids were compared, the expression profile of key genes involved in carotenoid biosynthetic and catabolic pathways was performed, and the differentially expressed genes were identified using “Piqiutao” (white fruit flesh) and its mutant yellow “Piqiutao” at different fruit development stages. The results showed that the total carotenoid content in yellow “Piqiutao” was remarkably higher than that of “Piqiutao,” and the accumulation of β-cryptoxanthin, α-carotene, and β-carotene was significantly different, which was most likely caused by the differential expression of CCD4. Therefore, CCD4 may be an essential gene that causes the yellow fruit flesh of yellow “Piqiutao.” However, the coding region sequence of CCD4 was entirely identical, and the intron was inserted by a retrotransposon in “Piqiutao” and its mutant, indicating that the expression difference was not caused by the sequence mutation and retrotransposon insertion.

Keywords Piqiutao · Mutant · Yellow flesh · Carotenoids · Expression profile · CCD4

Key message This study revealed that the differential expression of CCD4 was likely to be the essential reason for the significant difference in carotenoid content between “Piqiutao” and its yellow-flesh mutant, and the differential expression of CCD4 was not caused by the mutation of the coding region and promoter region of CCD4 and the retrotransposon insertion.
Introduction

Peach (Prunus persica) originates from western China and is the third most important temperate fruit tree after apple and pear. Peach flesh color is mainly divided into yellow, red, white, and cyan. The flesh color is a Mendelian genetic trait controlled by a pair of alleles, and white/yellow is controlled by the Y locus of the first linkage group, and white is dominant over yellow (Adami et al. 2013). As compared to white-flesh peach, yellow-flesh contains more abundant carotenoids. Carotenoids are mainly a class of C₄₀ terpenoids, which play an essential role in multiple biological processes such as photosynthesis, photomorphogenesis, photoprotection, and development of higher plants (Nisar et al. 2015). Meanwhile, carotenoids are also an indispensable part for human diet health and nutrition. They not only provide humans with important precursors of vitamin A but also help reduce the incidence of cardiovascular diseases, cancer, and blindness (Fiedor and Burda 2014).

Recently, with the development of molecular biology research methods, related genes in carotenoid biosynthesis pathway have been isolated and identified from bacteria and plants, which provides a useful tool for us to utilize genetic engineering to modify carotenoid content and composition. Phytoene synthase (PSY) is the most widely studied enzyme, as it is involved in the key rate-limiting step in the carotenoid synthesis pathway. In tomato fruit, carotenoid biosynthesis pathway is blocked due to activity loss of PSY1, resulting in a yellow mutation, while the missense mutation of PSY2 in cassava causes its roots to appear yellow and accumulate more abundant carotenoids (Welsch et al. 2010; Kachanovsky et al. 2012). Lycopene cyclization is the critical branch of the synthetic pathway, and lycopene β-cyclase (β-LCY) and lycopene ε-cyclase (ε-LCY) produce carotenoids with β-ring and ε-ring, respectively. Linear lycopene produces β-carotene under LCYB cyclization, whereas LCYE and LCYB act together to synthesize α-carotene. The relative expression of LYCB and LYCE determines the carotenoid flux, which affects the α/β-carotene ratio (Fu et al. 2019). The β-carotene content has been greatly improved after silencing LYCE by RNAi, and the overexpression of LYCB2 up-regulated carotenoid-related genes and increased β-carotene content in sweet potato (Kim et al. 2013; Kang et al. 2018). Finally, carotenoid cleavage oxygenases (CCOs) catalyze the degradation process of carotenoids, in which carotenoids are cleaved into various apocarotenoids by specifically cutting the conjugated double bond of C₄₀ carotenoids. In Arabidopsis, there are at least nine family members, four CCDs (CCD1, CCD4, CCD7, CCD8), and five NCEDs (NCED2, NCED3, NCED5, NCED6, and NCED9). Carotenoids produce various aromatic substances and phytohormones by CCDs and NCEDs.

Research has increasingly indicated that carotenoid accumulated in various plant species and tissues and was found to be negatively correlated with the expression of CCD4, suggesting its roles in carotenoid turnover. CCD4 gene was highly dynamic, which might contribute to diverse coloration in some plant species; for example, in chrysanthemum, the low expression level of CmCCD4a was tightly associated with the loss of the CmCCD4a in the genome and the loss of a function that normally enhanced CmCCD4a transcription (Yoshioka et al. 2012). In citrus, it was well documented that CCD4b was the major factor in determining orange reddish flavedo formation (Zheng et al. 2015). More recently, emerging evidence suggested that the presence of a putative 5’ cis-regulatory enhancer within an MITE transposon was responsible for the enhanced allelic expression of CCD4b in red-peeled citrus (Zheng et al. 2019). In Arabidopsis, the single nucleotide polymorphisms and insertions and deletions at the locus may be a critical reason for the expression difference of CCD4 (Gonzalez-Jorge et al. 2013). For peach, many researches have shown that CCD4 was a key gene controlling yellow/white trait, and its transcription level in yellow-flesh peaches was closely related to the insertion of a retrotransposon and a frame shift in the microsatellite sequences of the first exon (Fukamatsu et al. 2013). However, the transcription level ultimately determines the number of coding enzyme, which has an important impact on the biochemical process. The activity of the carotenoid metabolism–related enzymes usually is lost in extraction and separation process and is almost inactive in vitro, leading to the failure in accurately measuring their activity. As a result, it is unable to fully confirm the decisive effect of CCD4 on yellow/white trait through biochemical analysis (Giberti et al. 2019). Besides, reported findings are barely focused on the codig region variation of CCD4, which implies that evidences are inadequate and further studies are needed to better understand the fruit flesh coloration mechanism of peach.

In the summer of 2016, a natural mutant with yellow fruit flesh was discovered in “Piqiu Tao” with white fruit flesh, and the trait was stable in 3 years continuous observation (Fig. 1). These genotypes offer ideal experimental material for the study on fruit flesh coloration mechanism of peach.

In the present study, “Piqiu Tao” and its yellow mutant were used as research material to reveal the mechanism of coloration mutation from the physiological and molecular aspects, which provided the theoretical basis for the exploitation of carotenoid regulatory genes, the early identification of mutations, and was of great significance for the promotion and application of yellow-flesh peach in the future.
Materials and Methods

Plant Material and Sample Collection

“Piqiutao” and its mutant were planted in Wengong Town, Renshou County, Meishan City, Sichuan Province, China. The trees of the two cultivars were 9 years old, whose root-stocks were local wild peach trees. The field management methods, growth situation, and yield were consistent. Healthy fruits were collected successively at 15-day intervals from May 5, 2017, until July 5 (fruit ripening period). All sample was transferred immediately to the laboratory, and then the pulp was quickly plunged into liquid nitrogen and stored at −80 °C.

Carotenoid Extraction and Detection

Carotenoid extraction, carotenoid standard solution preparation, standard curve drawing and HPLC chromatographic conditions referred to the method of Yan et al. (2015), the total carotenoid content was determined by spectrophotometry (Yan et al. 2013a, b). Using Agilent 1260 high performance liquid chromatography system, DAD UV detector and YMC-C30 carotenoid analysis dedicated column (4.6 mm × 250 mm, 5 μm) were employed to detect the type and amount of carotenoids.

Real-Time Quantitative PCR Analysis of Carotenoid-Related Genes

The pulp total RNA at different development stages was extracted using a RNAprep Pure Plant Kit from Tiangen Biochemical Technology Corporation, and the first strand of cDNA was synthesized according to the PrimeScript™ RT reagent Kit (Perfect Real Time) from Takara. Carotenoid biosynthesis–related genes and reference gene of the peach were synthesized using the primers designed by Brandi et al. (2011), including two genes related to isoprenoid metabolism (DXS and HDR), eight genes related to carotenoid biosynthesis (PSY, PDS, ZDS, LYCB, LYCE, ZEP, CHYB, CHYE), four genes related to carotenoid metabolism (CCD1, CCD4, NCED1, NCED2), and actin gene (rps28). Real-time PCR was performed using TaKaRa’s TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus), and each sample was repeated three times. Then, the relative expression of carotenoid related genes was analyzed by 2^−ΔΔCT method.

CCD4 Allelic Genotype Verification

The cDNA of mature “Piqiutao” and its mutant was used as the template to clone the CCD4 coding region sequence. The genome DNA extracted with improved CTAB method was used to verify whether the retrotransposon had been inserted in the intron of CCD4. The PCR amplification primers were listed in Table 1.

Data Processing and Statistical Analysis

The carotenoid accumulation models and carotenoid synthesis–related gene expression patterns of the pulp of “Piqiutao” and its mutant were represented by GraphPad Prism 5.0. All values were shown as the mean ± standard errors. Statistical analysis was performed using the SPSS. Student’s unpaired t test was used to compare the means at p < 0.05.

Results and Analysis

Color Change of Peach Fruit in Different Development Stages

The flesh color change of “Piqiutao” and its mutant is shown (Fig. 2). In the first phase, the flesh of both

| Name               | Sequence                        |
|--------------------|---------------------------------|
| CCD4-F             | GTGAAGGGCAATAC                 |
| CCD4-R             | GGACACAATGACACTACAATT          |
| Retrotransposon-F  | AATACACCATTGAGCGTGAT           |
| Retrotransposon-R  | TGGAGCCCACCTAATTTCA            |
genotypes represented green color, and from the second phase, the flesh color showed coloration difference. “Piqiutao” faded green and gradually turned white, and the mutant turned from green to yellow and the color deepened by degrees.

**Carotenoid Accumulation Model**

Carotenoid composition and content analysis in the pulp were analyzed in Fig. 3. It showed that lutein, zeaxanthin, and β-carotene were predominant in both two cultivars. The content of lutein and zeaxanthin in the two cultivars was roughly similar, and both showed a gradual decline as the fruit matured. The β-carotene first rose then progressively degraded to disappear in the “Piqiutao,” but in the mutant, it gradually reduced to the lowest level and then increased to a maximum of 1.5 µg/gFW.

The accumulation pattern of β-cryptoxanthin and α-carotene varied greatly in the two cultivars. At the first stage, β-cryptoxanthin was both not detected, but in the mid-development stage, the β-cryptoxanthin content in “Piqiutao” was 0.23 µg/gFW and ultimately disappeared at the mature stage. With regard to mutant, β-cryptoxanthin began to accumulate from mid-term, whose change tendency was relatively stable, at about 0.28 µg/gFW. The α-carotene was not detected in “Piqiutao” throughout all stages, whereas the mutant constantly accumulated α-carotene from the mid-term and displayed a steady upward trend, reaching the highest content at maturity, about 0.97 µg/gFW.

During the fruit ripening process, the total content of carotenoids in “Piqiutao” continuously declined to a minimum of 0.99 µg/gFW, while the mutant behaved a gradual upward trend, reaching a maximum content of 14.08 µg/gFW at maturity, which was about 14-fold higher than “Piqiutao.”

**Carotenoid Synthesis–Related Gene Expression Pattern**

Carotenogenic gene expression pattern in the pulp is shown in Fig. 4. Among these genes, the expression trends of PSY, ZDS, LYCB, ZEP, and CHYB of “Piqiutao” coincided with the mutant, and among them, the ZDS expression abundance in mutant was remarkably higher than that of “Piqiutao” throughout all periods. However, the expression profiles of PDS, LYCE, and CHYE were slightly different. The PDS gradually increased and later decreased in “Piqiutao”; however, in the mutant, it first rose, a decline thereafter, then increased, and finally sharply dropped. LYCE was slowly up-regulated to third stage and then rapidly decreased to the lowest level in “Piqiutao.” As for the mutant, the highest expression abundance period was at the second stage, followed by an abrupt decrease to the lowest level.

NCED1 and NCED2 had a relatively similar dynamic change, both were in a stable low expression state in the early development stage and then rose sharply to the highest point in the late stage, and the expression level of mutant was extremely higher than that of “Piqiutao.” CCD1 in both genotypes appeared as a rapid increase, then a slight decrease, an abrupt rise, and an eventual decrease. Nevertheless, the expression pattern of CCD4 was absolutely distinct. “Piqiutao” initially was at low level, then slowly rose from the first stage to the third stage, after that sharply up-regulated to the highest level, and finally slightly decreased at maturity, while CCD4
was lowly expressed in the mutant, and its abundance was always markedly lower than that of “Piqiutao.”

**Identification of CCD4 Allelic Genotype**

The CCD4 coding region cloning primers were designed based on 5′ UTR and 3′ UTR regions, and sequencing results illustrated that the open reading frames (ORF) of CCD4 were 1794 bp in the two peaches, which belonged to the homozygous w<sup>1</sup> genotype.

The retrotransposon insertion verification primers were designed based on the first exon sequence and retrotransposon sequence, and the electropherograms indicated that there existed a retrotransposon in the intron of CCD4 of the two cultivars, and both were heterozygous for the y<sup>2</sup> genotype (Fig. 5).

**Discussion**

**Accumulation Difference of Carotenoid Leads to the Yellow-Flesh Mutant**

Researches on the peach carotenoid have shown that the primary portions were lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene, in which the β-carotene content was the highest, and the fruit color is mainly associated with the component and amount of carotenoids (Yan et al. 2013a,
b). In our study, lutein and zeaxanthin displayed uniform pattern and content in the two cultivars; therefore, they were not the major cause for the color divergence. In the pulp, β-cryptoxanthin and α-carotene started to accumulate from mid-development, and the content in the mutant was significantly higher than that of “Piqiutao.” Moreover, the β-carotene content in the mutant was also obviously higher than “Piqiutao” at maturity; therefore, the continuous accumulation of β-cryptoxanthin, α-carotene, and β-carotene in the mutant was a critical reason for the color distinction. However, Zhu et al. (2015) considered that the flesh color of “Zhongyoutao 9” mutant was chiefly associated with the accumulation of violaxanthin, β-carotene, and zeaxanthin, indicating that the accumulation pattern of carotenoid was diverse in peach varieties.

**Inactivation of CCD4 Leads to Carotenoid Accumulation in the Flesh of Mutant**

The abundant accumulation of β-cryptoxanthin, α-carotene, and β-carotene in the pulp of the mutant may be linked with the joint regulation of HDR, PSY, ZDS, LYCB, LYCE, and CHYE. The NCED1 and NCED2 expression patterns were inconsistent with the carotenoids accumulation, but CCD4 expression patterns were always low in the mutant and strongly expressed in “Piqiutao” at late stages. These results combined to suggest that the expression difference of CCD4 was the major determinant for the color mutation, which was coherent with the previous researches on the white/yellow pulp coloration mechanism of peach (Adami et al. 2013; Ma et al. 2014; Zhu et al. 2015; Bai et al. 2016; Cao et al. 2017; Zhu et al. 2018).

Higher plants primarily utilize carotenoid dioxygenase (CCD) and 9-cis-epoxy carotenoid dioxygenase (NCED) to...
cleave carotenoids into various apo-carotenoids, and CCD4 possesses the capacity of cleaving carotenoids located in the plastids into colorless substance, for the reason that its transcription levels are usually negatively correlated with carotenoid content (Ruiz-Sola et al. 2014). Song et al. (2017) assumed that the down-regulation of MdCCD4b was one of the major determinants for carotenoid accumulation at the ripening stage in the yellow-flesh apple. The CCD4 expression abundance in yellow azalea petals was much lower than that of white azalea petals during the growth process, and the CpCCD4a and CpCCD4b were highly expressed in white-flesh summer squash, which was negatively related to carotenoid content, and for wolfberry, CCD4 was highly expressed in petals and leaves, and its expression level was consistent with the β-carotene accumulation (González-Verdejo et al. 2015; Ureshino et al. 2016; Tian et al. 2017).

**CCD4 Multiple Variation Mechanisms in Different Plants**

CCD4 exists in multiple mutation mechanisms, leading to expressing distinctly between yellow and white tissues. Studies have discovered that white chrysanthemum mutated to yellow petals owing to the CmCCD4a deletion in the genome, by RNAi treatment, white chrysanthemum accumulated large amount carotenoid and turned into yellow petals (Ohmiya et al. 2006; Ohmiya et al. 2009; Jo et al. 2016). The yellow petal formation of *Brassica napus* was tightly concerned with the function loss of CCD4 as a result of the CACTA-like transposable element insertion in the coding region (Zhang et al. 2015). For *Osmanthus fragrans*, the differential expression of CCD4 was closely related to its promoter methylation degree and the regulation of OfWRKY3 transcription factor (Han et al. 2014; Han et al. 2016). In peach, there were at least three different variation mechanisms in *PpCCD4*, including the number difference of microsatellite repeats (TC)n, retrotransposon insertion in intron, and single nucleotide mutation, as a result, CCD4 cannot express normally, causing a yellow-/white-flesh color shift (Falchi et al. 2013). Zhu (2017) revealed that the microsatellite repeat (TC)n number variation played a key role in determining the low expression of CCD4 in the “Zhongyoutao 9” yellow-flesh mutant.

However, in this study, the coding region sequence of CCD4 was entirely identical, and the intron was inserted by a retrotransposon in “Piqiuato” and its mutant, which varied with former studies. The carotenoids accumulation pattern in plant tissues is regulated by various mechanisms, and the final amount is mainly determined by its biosynthesis, metabolism, and storage capacity (Nisar et al. 2015). Carotenoids are synthesized and stored in plastids, which suggested that plastid-related genes are a critical regulatory point for its accumulation levels, such as the Or discovered in cauliflower, the *PAP* associated with the white-flesh loquat formation, and fibrillin protein richened in red pepper (Lu et al. 2006; Simkin et al. 2007; Fu et al. 2012). Similarly, in this study, the appearance of yellow-flesh mutant may be partially linked with these genes. Certainly, transcription factor is also one of the important elements regulating the transcription level of CCD4. For example, Han et al. (2016) considered that OfWRKY3 had a positive regulatory effect on CCD4, and the expression divergence of CCD4 was responsible for the color difference in petals of “Dangui” and “Yingui.” Meanwhile, Ma et al. (2014) also found that the promoter region of CCD4 contained cis-acting elements associated with photoresponse and hormone response, which may have affected its temporal expression pattern.

**Conclusion**

Collectively, these results of this study elucidated that CCD4 expression difference had nothing to do with its sequence mutation and retrotransposon insertion, and there may exist other unknown regulatory mechanisms such as transcription factors and miRNAs. More importantly, results from the above further complemented the previous reported findings, and future investigations on the carotenoid regulation mechanism may contribute to profoundly understand the mutation causes. The discovery and research of the spontaneous mutant are of great significance for exploiting carotenoid regulatory genes and breeding new yellow-flesh peach varieties.

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