Materials and methods

1. Plant materials

Fruit were collected from five-year-bearing trees of Shiranui and Citrine Shiranui grown in Pujiang county, Chengdu city, Sichuan, China. In the sampled orchards, the flavedo color of the two cultivars breaks from October to late December. The fruit ripen in March (as indicated by the total soluble solid content in the fruit flesh of up to 10° Brix), during which the change in flavedo color is not obvious. Fruit sampled for the study were collected at the 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 full ripening stage (Stage VI). The fruit external appearance at each stage is shown in Fig. 1A. Ten fruit from one tree were mixed to represent one biological replicate. Three biological replicates at each stage were collected. The flavedo with 2 cm-width around a fruit equator was used.

2. Determination of total carotenoid content

The total carotenoid content was determined using a modification of the method described by Tao et al. (2007). The flavedo of Shiranui and Citrine Shiranui fruit was ground to powder in liquid nitrogen. A freeze-dried sample (1 g) of the powder was extracted with 10 mL pigment extraction solution (*n*-hexane:acetone:anhydrous ethanol, 2:1:1, v/v/v), containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant. The extracts were centrifuged for 5 min at 17 000 r/min at 4 °C. The supernatant containing the carotenoids was cleaned with saturated sodium chloride solution and recovered using a liquid separation funnel. The recovered supernatant was diluted with *n*-hexane to 10 mL, of which 1 mL was used for determination of total carotenoids using an enzyme-labeled UV/Vis microplate spectrophotometer at 450 nm (Multiskan GO, Thermo Scientific, Waltham, MA, USA).

3. Determination of carotenoid components

The remaining extract solution was saponified in the following steps. The extract was dried in a vacuum drier and then dissolved in 2 mL methyl tert-butyl ether (MTBE) containing 0.01% BHT. Two milliliters potassium hydroxide–methanol saturated solution containing 0.01% BHT was added to saponify the extract overnight. The saponified solution was purified with sodium chloride saturated solution and dried in a vacuum drier, then redissolved in 1000 μL acetone containing 0.01% BHT. The solution was centrifuged at 2000 r/min and the supernatant was filtered through a 0.22 μm organic filter membrane. The carotenoid components in the filtrate were analyzed with a high-performance liquid chromatography system (Shimadzu, Milan, Italy) equipped with a CBM-20A controller, two LC-20AD pumps, a DGU-20A3 degasser, a SIL-20AC autosampler, and an SPD-M20A photo diode array detector at 450 nm. The mobile phases comprised 90% (volume fraction) methanol solution (eluent A) and MTBE (eluent B). To prevent carotenoid

degradation on the column, 0.01% BHT was added to each eluent. The elution gradient was as follows: 0–5 min 20% eluent B, 5–8 min 35% eluent B, 8–13 min 55% eluent B, 13–22 min 65% eluent B, 22–24 70% eluent B, and 24–35 min 20% eluent B. The flow rate was 0.8 mL/min and the injection volume was 20 L. The column temperature was set at 25 ℃.

A total of eight individual carotenoids, such as *trans*-violaxanthin, 9-*cis*-violaxanthin, lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene, and β-carotene (all from Extrasynthese Company), were identified according to the retention time of the standard samples. Neoxanthin, phytoene, phtytofluene a/b and ζ-carotene were determined as previously reported (Hyoung, 2001). The proportion of an individual carotenoid component was determined by dividing the peak area of the carotenoid by the sum of all peak areas of carotenoids detected. Phtytofluene proportion were the sum of phtytofluene a and b.

4. Relative transcript level of carotenoid biosynthesis genes

The relative transcript levels of nine genes involved in carotenoid biosynthesis were analyzed, comprising *PSY*, *PDS*, *ZDS*, *LCYb*, *HYb*, *ZEP*, *NSY*, *LCYe*, and *HYe*. Total RNA was extracted from fruit pulp using TRIzol Reagent (Invitrogen, Shanghai, China). First-strand cDNA was synthesized using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed on a CFX96™ Real-Time System C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the NovoStart® SYBR qPCR SuperMix Plus Kit (Novoprotein, Shanghai, China). *Actin* was used as an internal reference gene. The relative transcript levels of the analyzed genes were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak et al., 2001). Three biological replicates were analyzed for each sample. The primers used are listed in Table S1.

5. Statistical analysis

One-way analysis of variance (ANOVA) and the Student-Newman-Keuls *q* test were performed at the 5% significance level with IBM SPSS Statistics 19.0 software (IBM Corporation, Armonk, NY, USA). Contrast analyses were used to test the differences between two cultivars at the same developmental stage.

Table S1 Primers for carotenoid biosynthesis genes

Gene	Gene name	Gene code	Former primer (5′-3′)	Reverse primer (5′-3′)

PSY	Phytoene synthase	Cs6g15910.4	TGTTGGTCAGGTACAGGTAAGATCA	CAATCGCTCACCCCATGAAC
PDS	Phytoene desaturase	AJ319760	ATAATTGGCGGACAGGCATA	CCTCTGTCGTCACTCGATCA
ZDS	zeta-Carotene desaturase	AF372617	ATCAGTGCTCGTTGTATGCTTACTAT	CCCTTGAGCATCCGCAAT
LCYb	Lycopene beta-cyclase 1	AY679168	GGCTATATGGTGGCAAGGACTT	CAGAATTGAGGCTTCGAACGA
HYb	beta-Carotene hydroxylase	AB114661	TTTGGGATGGCCTACATGTTC	GGCACGTCGGCAATGG
ZEP	Zeaxanthin epoxidase	AB114662	GAAGCAATTCTTCGACGTGACA	ACCGAGTCCCCAAGCAAAGT
NSY	Neoxanthin synthase	orange1.1g010693m	GCAAGTCTCATCGCGTCATA	AAAACTTGGCCGATGACTTG
LCYe	Lycopene epsilon-cyclase	AY533827	CAACTGGATATTGAGGGCATCA	CAAGGAAACCGTGCCACATC
HYe	Carotenoid epsilon hydroxylase	orange1.1t01082.1	ATGCGTCTCTACCCACATCC	ACCATTCCTCACAGGTCTGG