

Article

Divergent Effects of Distinct *SlCRTISO* Alleles on Phenolic Content, Fruit Size, and Transcriptomic Profiles in *Tangerine* Tomatoes

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Highlights

What are the main findings?

- Distinct *SlCRTISO* alleles drive divergent fruit quality-related metabolic and transcriptomic changes in tangerine tomatoes.
- ABA levels are negatively correlated with phenolic compound accumulation.

What are the implications of the main findings?

- *SlCRTISO* alleles differentially influence both primary and secondary metabolic pathways.
- A trade-off between nutrition and fruit size exists among tangerine tomatoes carrying different *SlCRTISO* alleles.

Abstract

Carotenoids are essential nutritional compounds in tomato (*Solanum lycopersicum*), and mutations in carotenoid biosynthesis genes can alter both fruit color and other quality traits. Carotenoid isomerase (*CRTISO*) converts polyycopene to all-*trans*-lycopene; its mutation results in the orange-fruited *tangerine* mutant characterized by polyycopene accumulation. However, the broader impacts of distinct *SlCRTISO* mutations on fruit quality traits and agronomic performance remain largely unexplored. Here, we evaluated agronomic traits and nutritional quality in two *tangerine* mutants carrying distinct *SlCRTISO* alleles. The *yellow/orange-fruited tomato 5* (*yft5*) mutant, which harbors a single point mutation in *SlCRTISO*, exhibited lower flavonoids, chlorogenic acid, and polyycopene than the CRISPR/Cas9-mediated knockout mutants, but maintained fruit size and weight comparable to wild type, whereas knockout lines showed severely reduced fruit size, highlighting the potential agronomic advantage of *yft5*. Pearson correlation analysis revealed negative associations between abscisic acid (ABA) levels and rutin, chlorogenic acid, as well as the polyycopene-to-lycopene ratio during fruit ripening. Transcriptome analysis demonstrated that knockout mutations more profoundly affected primary metabolism than the *yft5* point mutation. Notably, *9-cis-epoxycarotenoid dioxygenase 2* (*NCED2*), a gene involved in ABA biosynthesis, was specifically upregulated in *yft5* fruits at the mature green stage (38 days post-anthesis (dpa)), consistent with ABA levels. Quality-related genes including *chalcone synthase* (*CHS*), *citrate synthase* (*CS*), and *invertase* (*INV*) were significantly upregulated in knockout lines during ripening. These findings reveal a trade-off between phenolic content and fruit size in *tangerine*

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tomatoes carrying distinct *SlCRTISO* alleles, positioning *yft5* as a valuable genetic resource for breeding high-quality varieties.

Keywords: *tangerine* tomato; *SlCRTISO*; *Solanum lycopersicum*; prolycopene; flavonoids; abscisic acid (ABA); transcriptome

1. Introduction

Tomato (*Solanum lycopersicum*) fruit ripening is a coordinated process accompanied by chlorophyll degradation and the accumulation of carotenoids and flavonoids, leading to dramatic changes in fruit color [1]. Carotenoids, flavonoids and other compounds such as sugars, vitamin C and phenolic acids serve as essential nutrients or energy sources beneficial to human health [2]. The consumption of tomato fruits has been associated with antioxidant activity, cardiovascular protection, and cancer prevention [3].

As a climacteric fruit, tomato has been extensively studied for its pigment accumulation and phytohormone change during ripening. It has been demonstrated that abscisic acid (ABA) is synthesized before ethylene emission at the on-set of fruit ripening in climacteric fruit [4,5]. ABA alone is unable to promote fruit ripening or cause color change in the absence of ethylene [6]. However, alterations in the expression of genes involved in ABA biosynthesis and signaling can significantly influence the accumulation of carotenoids and flavonoids. Overexpression of *abscisic acid-responsive element binding protein 1* (*AREB1*) enhances anthocyanin levels in tomato fruits [7], while silencing *9-cis-epoxy-carotenoid dioxygenase 1* (*NCED1*) leads to increased lycopene accumulation as a consequence of elevated ethylene production [8].

Carotenoids are synthesized via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in higher plants and serve as precursors for important phytohormones, including ABA and strigolactone [9–11]. In horticultural plants, mutations in carotenogenesis-related genes often cause diverse phenotypes affecting quality traits beyond color change. For instance, the tomato *zeaxanthin epoxidase* (*ZEP*) mutant *high-pigment 3* exhibits reduced ABA levels and accumulates more pigments in fruits [12]; overexpression of *phytoene synthase 1* (*PSY1*) gene causes dwarfism in tomato plants due to gibberellin deficiency [13]; silencing of upstream carotenogenesis genes induces pre-harvest spouting owing to ABA [14]; suppression of ζ -carotene desaturase (*ZDS*) leads to reduced total brix and ABA levels [15]; and *carotenoid isomerase* (*CRTISO*) loss-of-function mutants display reduced drought sensitivity and impaired endomycorrhizal colonization in tomato roots, as well as increased tiller number in rice (*Oryza japonica*), due to deficiencies in ABA and strigolactone [16,17].

Among these carotenogenic enzymes, *CRTISO* occupies a unique position, catalyzing the final step before cyclization—the conversion of 7,9,7',9'-tetra-cis-lycopene (prolycopene) to all-trans-lycopene—and thus directly determining the flux toward downstream carotenoids and their derived hormones. *CRTISO* is also known as the rate-limiting enzyme for the biosynthesis of lutein, which is important for photoprotection [18], thus the *CRTISO* loss-of-function mutants exhibit perturbed photomorphogenesis and damaged chloroplast development in leaves [19,20]. In rice, *OsCRTISO* mutations adversely affect both plant growth and crop yield due to reduced photosynthetic rates and increased susceptibility to photo-oxidation, leading to shorter panicles and fewer grains [21–23]. In contrast, *CRTISO* loss-of-function mutants in tomato, known as *tangerine*, over-accumulate prolycopene and other upstream *cis*-carotenes in ripe fruits without visible photodamage [15]. Lycopene, the most abundant carotenoid in tomatoes, possesses excellent antioxidant and biological activities and has been widely applied in the

healthcare field [24]. Notably, the polycopene, as a *cis*-isomer of lycopene accumulated in *tangerine* tomatoes, exhibits higher nutritional value owing to its greater bioavailability compared to all-*trans*-lycopene [25–28]. Several *tangerine* tomato lines with distinct *SICRTISO* mutations have been characterized in various genetic backgrounds, including Ailsa Craig (*tangerine*³¹⁸³), Micro-Tom (*tangerine*^{mic}), M82 (*tangerine*³⁰⁰²) and Arka Vikas (*tangerine*⁴⁰⁶), all of which exhibit varying degrees of polycopene accumulation [29–31]. However, most studies have focused on carotenoid composition changes and the biological activity conferred by *cis*-lycopene, while few have investigated the nutritional quality and agronomic performance of these cultivars [16,32,33]. Beyond carotenoids, other fruit quality attributes such as flavor, brix/acid ratio, and flavonoids are also critical determinants of tomato nutritional value and consumer preference. The brix-to-acid (brix/acid) ratio, largely determined by the balance between sugars (primarily glucose and fructose) and organic acids (mainly citric acid), is a key index of fruit flavor quality [34,35]. Flavonoids, including rutin and chalconaringenin, are major phenolic antioxidants in tomato fruits and contribute significantly to their health-promoting properties [36]. These quality traits are regulated by complex metabolic networks that can be influenced by genetic variation in carotenoid biosynthesis genes, yet the extent to which *CRTISO* mutations affect these attributes remains poorly understood. Given that carotenoid metabolism is tightly linked to phytohormone biosynthesis (particularly ABA) and that perturbations in this pathway can trigger broad transcriptional reprogramming, we hypothesized that distinct *SICRTISO* mutations differentially affect fruit quality traits beyond carotenoid accumulation, and that the severity of the mutation correlates with the extent of metabolic and global gene expression patterns.

To fill the knowledge gap and confirm the hypothesis, in this study, we evaluated the agronomic traits and nutritional quality of fruits from two types of *SICRTISO* (*tangerine*) mutants in the background of cultivar P86 (*Solanum lycopersicum*). P86 is an indeterminate, high-water-content edible tomato that produces larger fruits than the commonly used Ailsa Craig (AC) cultivar, making it well suited for assessing fruit quality traits. One of the *tangerine* mutants is the ethyl methanesulfonate (EMS)-induced *yellow/orange-fruited tomato 5* (*yft5*) mutant, which carries a T2852C mutation in the seventh exon of *SICRTISO*, resulting in a W382R missense mutation in the encoded protein. The other consists of *SICRTISO* knockout lines (*YFT5-KO*) generated by CRISPR/Cas9 gene editing, which carry frameshift mutations leading to premature translation termination. These allelic series allow us to distinguish between the effects of partial versus complete loss of *CRTISO* function on fruit quality traits. Because *CRTISO* functions upstream of ABA biosynthesis, we reasoned that the severity of the mutation would correlate with the degree of transcriptional and metabolic reprogramming, with the knockout lines expected to exhibit more extensive changes than the *yft5* point mutant. We compared carotenoid composition, fruit size, brix/acid ratio and phenolic compounds between these two mutants, and analyzed the correlation between ABA levels and nutritional indices across three fruit developmental stages. Furthermore, we performed transcriptomic analysis of four key pathways associated with these quality traits to provide a deeper interpretation of the phenotypic differences between the two mutant types. This integrated evaluation is essential for selecting optimal *SICRTISO* alleles for future breeding, enabling the full realization of the bioavailability advantages of *tangerine* tomatoes without compromising agronomic performance, as observed in previous mutants [32].

2. Materials and Methods

2.1. Plant Growth Conditions

Seeds of wild type (*S. lycopersicum*, cv. P86) were kindly provided by Dr. Wang Fu (Qingdao Agricultural University, Qingdao, China). The mutant *yellow/orange-fruited tomato 5* (*yft5*) was created from P86 by ethyl methanesulfonate (EMS) mutagenesis. T₂-generation seeds were collected from T₁ knockout lines (*YFT5-KO*) that were confirmed to carry homozygous frameshift mutations in *SICRTISO*. Tomato plants were grown in ground soil from March to June in a drip-irrigated, naturally ventilated greenhouse at Shanghai Jiao Tong University, Shanghai, China (121°30'10.89" E, 31°3'5.20" N, altitude 5 m). Environmental conditions, including light (approximately 12–14 h of daylight), temperature (ranging from approximately 20 to 40 °C), and humidity, followed natural seasonal fluctuations without artificial regulation. Standard horticultural practices were applied for fertilization and pest management.

2.2. Vector Construction

The CRISPR/Cas9 editing recombinant vector *pYFT5-KO* was constructed using the empty vector *pTX041* as the backbone, which was kindly provided by Professor Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China). The two editing targets were designed at the first and seventh exon of *SICRTISO* (*Solyc10g081650*) using online tools CRISPR-P v2.0 (<http://cbi.hzau.edu.cn/CRISPR2/>, accessed on 5 August 2026). The two 20 bp target sequences were 5'-GCAGCTTTGTGGCTTGAGTT-3' (exon 1) and 5'-GCTACCAGATGGGATACTTT-3' (exon 7). These two target sequences were then assembled into a single fragment by PCR amplification using plasmid *pTX043* (previously constructed recombinant vector) as a template. The amplified fragment with target sequences and homology arms were constructed to *pTX041* digested with *Bsa* I by homologous recombination.

The PCR amplification enzyme used for vector construction were rTaq DNA polymerase (Takara, Dalian, China). The primers used for amplification are CRTISO-cris-F: 5'-gaatctaacagtgtagtttgcagcttggcttgagttgttttagagctagaataagc-3' and CRTISO-cris-R: 5'-gc-tatttctagctctaaaacaagtatccatctggtagcaaaactacactgtagattc-3'.

2.3. Transgenic Generation

Transgenic plants were generated according to [37,38]. Briefly, cotyledons and hypocotyls of tomato seedlings from P86 were transformed with *A. tumefaciens* (strain EHA105) containing *pYFT5-KO* recombinant vector. After the plant tissue culture, the T₀ generation of regenerated transgenic seedlings were screened for positive lines by PCR amplification. The mutations at target sites were determined by Sanger sequencing and analyzed using DsDcodeM [39]. Seedlings carrying frameshift mutations in the *SICRTISO* gene that cause premature translational termination were transplanted into soil and allowed self-pollination to generate T₁ seeds.

2.4. *yft5* Mutation Identification

The *yft5* mutant was isolated from an ethyl methanesulfonate (EMS)-mutagenized population of the tomato cultivar P86. Map-based cloning delimited the causal locus to a region on chromosome 10 containing *SICRTISO* (*Solyc10g081650*). Sequencing of the candidate gene revealed a single T2852C point mutation in the seventh exon of *SICRTISO*, which is predicted to cause a W382R missense substitution in the encoded protein. This mutation co-segregated with the tangerine fruit phenotype in an F₂ mapping population. No nucleotide changes were detected in the other genes within the mapped interval,

none of which are predicted to be involved in carotenoid biosynthesis. Functional complementation analysis further supported that the mutant phenotype is attributable to this *SICRTISO* mutation. Candidate gene sequencing is conducted as described below. The full-length *SICRTISO* gene was amplified using the genome DNA extracted from *yft5* and P86 leaves as templates and subjected to sequencing. The mutation site was identified by pairwise alignment of two DNA sequences. The total RNA extracted from fruits at 43 dpa (days post-anthesis) using the RNAprep Pure Plant Kit (TIANGEN, Beijing, China) was used for cDNA synthesis by PrimeScript™ IV 1st strand cDNA Synthesis Mix (6215A, Takara, Dalian, China). The full-length cDNA of *SICRTISO* was obtained by PCR amplification using primers C-5'end-F: 5'-gttggtggtgtctgtaatcttg-3' and C-3'end-R: 5'-gtgattctatactggaggaac-3'. The PCR products were then cloned into a pLB vector using a lethal based fast cloning kit (VT205, TIANGEN, Beijing, China) for sequencing and mutation detection.

2.5. Agronomy Traits Assessment

Fruits from wild type (cv. P86), mutant *yft5* and *YFT5-KO* lines were collected at 38, 43, and 55 dpa, which corresponds to mature green (MG), breaker (BR) and red ripe (RR) stage of fruit ripening. After harvest, fruits were kept at room temperature for 2 h to allow recovery from wounding stress before measurement of physiological parameters. Ripe fruits at 55 dpa were used for agronomy traits assessment. Fruit weight, transverse diameter, and vertical diameter were measured from ten individual fruits per genotype. Brix, acidity, and Brix/acid ratio were determined from three to five individual fruits per genotype. Fruit hue value was measured from three individual fruits per genotype. All fruits were collected from different individual plants. The fresh fruit weight was measured using electronic balance (METTLER TOLEDO, Shanghai, China). The transverse and vertical diameter of fruits were measured using vernier caliper. The fruit brix and titratable acid was measured using handheld Brix-acid meter (PAL-BX/ACID 3, ATAGO, Tokyo, Japan). The color difference in fruits was measured using the portable colorimeter (CR-400, Konica Minolta, Inc., Tokyo, Japan). The hue value was calculated based on the L, a and b value provided by the colorimeter using the formula as follows [40]:

$$\text{Hue} = \tan^{-1}(b/a), a > 0, b \geq 0 \quad (1)$$

The hue value determines the basic colors including pure red, orange, yellow, and pure green, which are represented in hue value as 0°, 45°, 90° and 180°, respectively.

2.6. Experimental Design and Biological Replicates

For carotenoid measurement, phenolic compound quantification, ABA detection, and RNA extraction, pericarp tissue from the equatorial region of fruits harvested at 38, 43, and 55 dpa was sampled and immediately frozen in liquid nitrogen. Three independent biological replicates were prepared, each consisting of 3–4 fruits pooled from different individual plants. All samples for a given analysis were processed in a single batch.

2.7. Carotenoid Content Measurement

For carotenoid analysis, carotenoids extraction was conducted as follows under dim light. Briefly, frozen tomatoes were ground and 500 mg of the tissue powder was placed in a 15 mL tube. Then, 3 mL Tris-HCl buffer (Tris 50 mM, NaCl₂ 1 M, PH = 7.5) was added and vortexed for 10 s. Subsequently, 3 mL of extraction solvent (Chloroform:methanol = 2:1) was added, and the mixture was shaken on ice for 10 min. The extracts were then centrifuged for 10 min at 6000× g and 4 °C. The lower phase was transferred to a new 2 mL tube using a 1 mL syringe for carotenoid analysis. The organic

extract was dried using an SpeedVac Vacuum Concentrator (SPD2010, Thermo Fisher Scientific, Waltham, MA, USA) and redissolved in 1 mL of methyl *tert*-butyl ether (MTBE). The resulting suspension was centrifuged at 12,000× *g* for 10 min at 4 °C, and an aliquot of 200 µL of the supernatant was transferred to a UPLC vial for injection.

Carotenoids were examined using a ACQUITY UPLC I class/Vion IMS QTOF with a photodiode array (PDA) detector (Waters, Milford, MA, USA). Separation was performed on a BEH C18 column (dimensions, 2.1 × 100 mm, 1.7 µm; Waters) maintained at 45 °C. The mobile phases consisted of solvent A (ddH₂O with 0.1% formic acid), and solvent B (methanol: acetonitrile: isopropanol = 2:2:1); flow rate: 0.4 mL/min. Sample temperature: 10 °C. Detection wavelength: 450 nm. The injection volume was 1 µL. The mobile phase gradient was programmed from 30% A and 70% B to 0% A and 100% B. The all-*trans*-lycopene was identified using the standard purchased from Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China. The 7,9,7',9'-*tetra-cis*-lycopene (prolycopene) were identified depending on its absorption spectrum according to previous studies [41]. According to the molar absorption coefficient (ϵ_{mol}) in hexane, the estimated concentration of prolycopene/lycopene could be derived from their peak area according to the formula described in Anwar et al. (2022) [42].

2.8. Phenolic Compounds and Citric Acid Quantification

Phenolic compounds extraction was performed according to Muir et al. (2001) [43] with some modifications. Ground tomato powder (400 mg) was transferred to a 15 mL tube containing 70% methanol. The mixture was thoroughly vortexed and then sonicated for 30 min in an ice-water bath to prevent overheating. After extraction, the samples were stored at 4 °C overnight before UPLC-MS analysis. UPLC-MS was carried out using Acquity UPLC I class/Vion IMS QTOF (Waters, Milford, MA, USA). Separation was performed on a BEH C18 column (dimensions, 2.1 × 100 mm, 1.7 µm; Waters) maintained at 45 °C. The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The mobile phase gradient was programmed as follows: 95% A and 5% B initially, changed to 80% A and 20% B, and then to 0% A and 100% B. The injection volume was 1 µL, and the flow rate was 0.4 mL/min. Mass spectrometric detection was performed in negative ion electrospray ionization (ESI[−]) mode with the collision energy of 6 eV/20–45 eV. Four standards including rutin, chalconaringenin, chlorogenic acid and citric acid were purchased from Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China. They were used for identification and quantification of phenolic compounds (rutin, naringenin-chalcone and chlorogenic acid) and citric acid by generating standard curves based on their concentration and response value.

2.9. ABA Detection

For ABA extraction, the procedures were similar to Chai et al. (2018) [44] with some modifications. In brief, ground fruit powder (250 mg) was transferred to a 15 mL tube containing 2.5 mL of extraction solvent (isopropanol:H₂O:concentrated HCl = 2:1:0.002, *v/v/v*), vortexed vigorously, and then placed on a shaker at 4 °C with shaking at 100 rpm for 30 min. Subsequently, 2.5 mL of dichloromethane was added, and the mixture was returned to the shaker for another 30 min under the same conditions. After centrifugation at 1500× *g* for 5 min at 4 °C, the lower phase was collected using a 5 mL syringe and transferred to a new 15 mL tube for storage. An aliquot of 1 mL of the stock was then transferred to a 2 mL tube, evaporated to dryness, and redissolved in 200 µL of methanol for UPLC analysis using an UHPLC 30A/Sciex Quadrupole 5500 (Waters, Milford, MA, USA). ABA standard purchased from Shanghai LABLEAD Trading Co., Ltd. (Shanghai, China) was used for ABA identification and quantification based on the peak area and

concentration.

2.10. RNA-Sequencing and Transcriptome Analysis

For RNA-seq analysis, total RNA was extracted from the fruits of P86, *yft5* and *YFT5-KO19* at 38, 43 and 55 dpa using the TRIzol Reagent according to the manufacturer's instructions ($n = 3$). The RNA integrity was evaluated using a 5300 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and the RNA concentration was estimated using the ND-2000 NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA ($>1 \mu\text{g}$) having a RQN value ≥ 9 , concentration $\geq 20 \text{ ng}/\mu\text{L}$ and high quality ($\text{OD}_{260}/\text{OD}_{280} = 1.8\text{--}2.2$, $\text{OD}_{260}/\text{OD}_{230} \geq 2$) was used for the cDNA library preparation (Illumina Stranded mRNA Prep, Ligation from Illumina, San Diego, CA, USA), and quality was checked using Qubit 4.0 (Thermo Fisher Scientific, Waltham, MA, USA). The RNA-seq was performed by Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China) using NovaSeq $\times$ Plus sequencer ($2 \times 150 \text{ bp}$ read length) using NovaSeq Reagent Kit. The clean reads were obtained by trimming raw paired end reads and quality-controlled by Fastp with default parameters [45], including automatic adapter detection and removal, exclusion of reads lacking inserts due to adapter self-ligation, trimming of low-quality bases at the 5' end (Phred score < 20 , 4 bp sliding window) and at the 3' end (Phred score < 3), filtering of reads with more than five unknown nucleotides (N), and removal of reads shorter than 30 bp after trimming. The raw reads are available at <https://www.ncbi.nlm.nih.gov/sra/PRJNA1483294> (accessed on 5 August 2026), under BioProject ID: PRJNA1483294. HISAT2 was used to align clean reads to the reference genome (https://solgenomics.net/ftp/genomes/Solanum_lycopersicum/Heinz1706/annotation/ITAG4.0_release/, accessed on 5 August 2026) [46], and StringTie was used to assemble the mapped reads [47]. The TPM (the transcripts per million reads) was calculated to show the expression level of each transcript. The gene abundance was quantified by RSEM [48]. Differentially expressed genes (DEGs) analysis was performed using the DESeq2 [49], and the genes were prioritized with $|\log_2\text{FC}| \geq 1$ and $P_{\text{adj}} < 0.05$.

For gene functional enrichment analysis of DEGs, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was carried out at Benjamini-Hochberg-corrected adjusted p value < 0.05 , using Python scipy software in version 3.7 [50]. For cluster analysis, $\log_{10}$ TPM values of totally 3249 DEGs were clustered based on the Euclidean distance clustering algorithm. The Principal Component Analysis (PCA) was conducted using permutational multivariate analysis of variance (PERMANOVA) based on the TPM value in each sample [51].

2.11. Total RNA Extraction and Gene Expression Analysis

Total RNA from the frozen fruit samples of all lines was extracted using the RNAPrep Pure Plant kit (DP432, Tiangen, Shanghai, China) ($n = 3$). cDNA was synthesized from 500 ng of total RNA using PrimeScriptTM IV 1st strand cDNA Synthesis Mix (6215A, Takara, Dalian, China). Real time-quantitative PCR (RT-qPCR) was conducted using TB green premix Ex TaqTM II (RR420A, Takara, Dalian, China) and run on RTX384 real-time PCR detection system (Biorad, Shanghai, China) using the three-steps-program. The relative expression level of *NCED2* (*Solyc08g016720*) were normalized against that of the tomato *ACTIN* gene (*Solyc03g078400*). The relative expression level was calculated as $2^{-\Delta\text{CT}}$, and was averaged using three biological replicates. The following primers were used for qRT-PCR: *NCED2* (forward: 5'-tggttttcatgggacattcattagc-3', reverse: 5'-atctcccttctcaactccctattcc-3') and *Actin* (forward: 5'-ttgctgaccgtatgagcaag-3', reverse: 5'-ggacaatggatggaccagac-3').

2.12. Data Processing

All statistical analyses were performed using SPSS 25 software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA: IBM Corp.). Prior to analysis, data were tested for normality using the Shapiro–Wilk test. Homogeneity of variance was assessed using Levene’s test as part of the analysis of variance (ANOVA) procedure. One-way ANOVA followed by Duncan’s post hoc test was used to conduct multiple comparisons to determine group differences among genotypes. Student’s *t*-tests was used to perform pairwise comparisons between the gene-modified tomato lines and wild type. The grouped columns or lines with means and standard deviations, as well as the heatmaps were generated using GraphPad Prism 9.5 software (Boston, MA, USA, www.graphpad.com, accessed on 5 August 2026). The Pearson correlation coefficient (*r*) was calculated at *p* < 0.05 using Origin software (Origin, Version 2024. OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. The Point Mutation or Knockout of *SICRTISO* Gene Leads to Altered Carotenoids Composition

The *yft5* mutant in our study carries a T2852C point mutation in *SICRTISO* gene, leading to a W382R missense mutation in the encoding protein sequence. It exhibits an orange fruit color typical of *tangerine* tomato mutants [29–31] (Figure 1A,B). Two distinct *SICRTISO* knockout lines (*YFT5-KO*) generated by CRISPR/Cas9 gene editing produced truncated *SICRTISO* proteins due to the premature translation termination caused by frameshift mutation. They also possessed orange ripe fruits with the hue value closer to orange compared to *yft5* (Figure 1A–C).

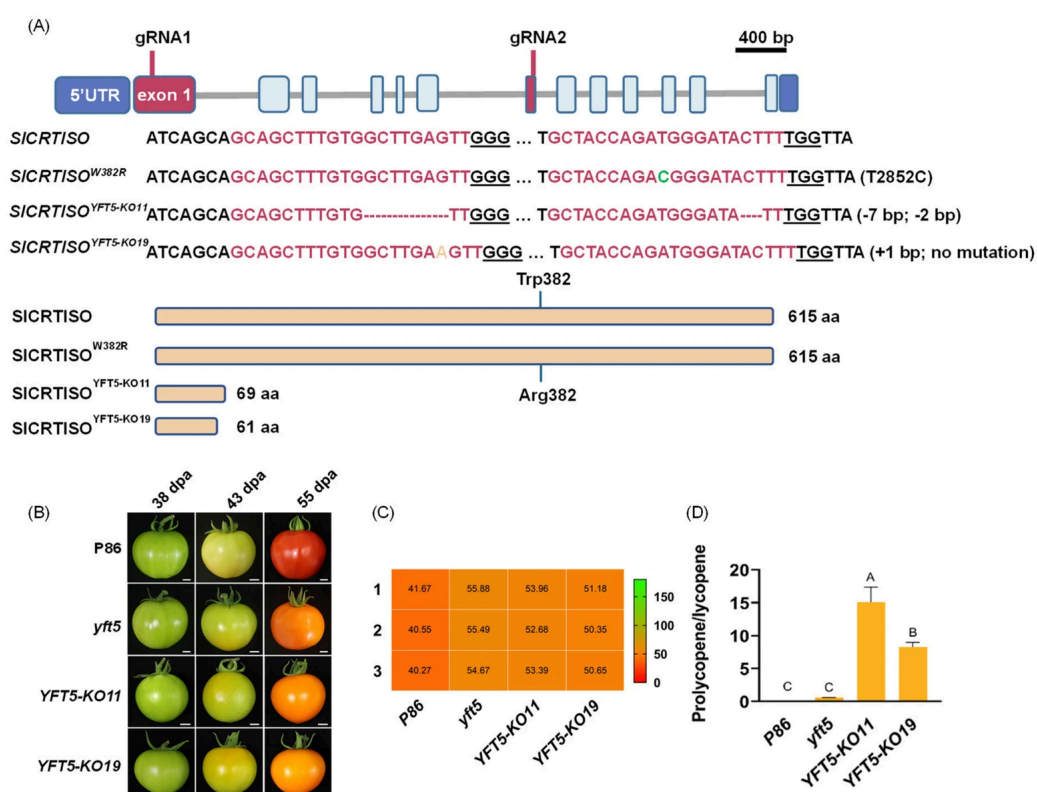


Figure 1. The phenotypes of mutant *yft5* and CRISPR/Cas9 editing lines. (A) The DNA and protein sequences of *SICRTISO* alleles resulting from the CRISPR/Cas9 editing (*SICRTISO^{YFT5-KO11}* and *SICRTISO^{YFT5-KO19}*) or point mutation (*SICRTISO^{W382R}*). Two targets were designed at the first and

seventh exon marked in red. The captain letters in red represent two target sequences. The underlined black letters represent the PAM (protospacer adjacent motif) sequences. The annotations in the parentheses represent the editing results. “-” represents deletion. “+” represents insertion. The green “C” represents the mutated 2852nd cytosine from thymine. The yellow rectangles represent the length of protein sequences translated from the mRNA with a premature stop codon or a single nucleotide substitution. The dark blue rectangles represent the 5'- or 3'-untranslated region. The light blue rectangles represent the exon. The gray lines represent the intron. gRNA, guiding RNA. Scale = 400 bp. (B) Phenotypes of tomato fruits in wild type (cv. P86), mutant *yellow/orange-fruited tomato 5* (*yft5*) and two CRISPR/Cas9 knockout lines (*YFT5-KO11* and *YFT5-KO19*) at 38, 43 and 55 dpa. Bars = 1 cm. Dpa, days post-anthesis. (C) The heatmap reflecting the hue values of fruit color at 55 dpa in different plant lines. (D) The concentration ratio of prolycopene to lycopene (prolycopene/lycopene) in mutant *yft5* and *YFT5-KO* lines at 55 dpa. Data are shown as means $\pm$ SD from three independent biological replicates and were analyzed by one-way ANOVA followed by Duncan's test at $p < 0.01$. Different uppercase letters indicate statistically significant differences between groups.

Carotenoid analysis of ripe fruits (55 dpa) revealed a considerably larger concentration ratio of prolycopene to lycopene in two *YFT5-KO* lines than mutant *yft5*, which was 25-fold and 14-fold of *yft5* in *YFT5-KO11* and *YFT5-KO19*, respectively. Conversely, no prolycopene was detected in P86 (Figure 1D). Both truncated proteins lack the conserved NAD/FAD(P)H-binding domain (normally starting at residue 97) and thus represent functionally equivalent null alleles. No exonic off-target mutations were predicted. The difference between the two knockout lines may therefore reflect clonal variation from independent regeneration events or subtle genetic background effects on carotenoid metabolism. These results indicate that *SlCRTISO* knockout leads to more prolycopene accumulation in ripe fruits than T2852C point mutation.

3.2. The Two Types of Mutations in *SlCRTISO* Differently Affect the Agronomy Traits and Nutritional Quality of Fruits

To investigate the effect of *SlCRTISO* mutation on more agronomy traits, the fresh weight, size and brix/acid ratio of ripe fruits were examined. The results showed that the fruit weight of *YFT5-KO11* and *YFT5-KO19* displayed significant reduction compared to the wild type, which decreased to approximately 74.6% and 69.7% of wild type, respectively (Table 1). Fruit size also decreased accordingly in the two *YFT5-KO* lines, with a more significant reduction in transverse diameter. In contrast, the reductions in fruit size and weight were relatively milder in the *yft5* mutant than in the *YFT5-KO* lines. The fruit weight of *yft5* reached 86.2% of the wild type, and its transverse diameter was closer to that of the wild type. In terms of fruit flavor, both *YFT5-KO* lines and *yft5* exhibited a higher brix/acid ratio than the wild type, driven by higher brix content and lower acidity, with *yft5* showing the highest ratio (Table 1).

Table 1. Agronomy traits of ripe tomato fruits from different lines ¹.

Plant Lines	Fruit Weight (g)	Transverse Diameter (cm)	Vertical Diameter (cm)	Brix (°BX)	Acid	Brix/Acid
P86	120.62 $\pm$ 19.64 ^a	61.46 $\pm$ 3.25 ^a	57.07 $\pm$ 2.61 ^a	3.34 $\pm$ 0.32 ^a	1.15 $\pm$ 0.13 ^a	2.91 $\pm$ 0.14 ^b
<i>yft5</i>	103.99 $\pm$ 25.15 ^{ab}	58.54 $\pm$ 5.02 ^{ab}	54.08 $\pm$ 5.17 ^a	3.70 $\pm$ 0.36 ^a	0.94 $\pm$ 0.37 ^a	4.29 $\pm$ 1.61 ^a
<i>YFT5-KO11</i>	89.95 $\pm$ 23.79 ^{bc}	54.84 $\pm$ 4.78 ^b	55.77 $\pm$ 3.37 ^a	3.64 $\pm$ 0.22 ^a	1.22 $\pm$ 0.28 ^a	3.10 $\pm$ 0.62 ^b
<i>YFT5-KO19</i>	84.07 $\pm$ 12.17 ^c	55.78 $\pm$ 3.09 ^b	49.65 $\pm$ 2.07 ^b	3.76 $\pm$ 0.34 ^a	0.98 $\pm$ 0.24 ^a	3.48 $\pm$ 0.29 ^{ab}

¹ The weight, transverse diameter and vertical diameter of fruits were measured from ten individual fruits per genotype from different plants. Brix, acidity, and brix/acid ratio were measured from

three to five individual fruits per genotype from different plants using a PAL-BX|ACID meter (ATAGO, Tokyo, Japan). Data were analyzed using one-way ANOVA followed by Duncan's test at $p < 0.05$. Different lowercase letters indicate statistically significant differences between groups.

Apart from carotenoids, other nutritional compounds including flavonoids, chlorogenic acid, and citric acid were detected. The citric acid content in *YFT5-KO* lines and *yft5* was lower than the wild type, consistent with the acidity measured by a handheld Brix-acid meter. However, the content of chlorogenic acid, the predominant phenolic acid in tomato fruits [52], was decreased in *yft5* and *YFT5-KO19*, but increased in *YFT5-KO11* (Table 2). Regarding the two main flavonoids in tomato, rutin and chalconaringenin [43], their content was dramatically higher in two *YFT5-KO* lines compared to the wild type, increased by 1.69- and 2.26-fold for rutin, and 4.90- and 6.60-fold for chalconaringenin, in *YFT5-KO11* and *YFT5-KO19*, respectively. The total flavonoid content remained unchanged in *yft5* compared to the wild type, with increased rutin but decreased chalconaringenin content. These results demonstrated that both knockout and T2852C point mutation in *SICRTISO* improved the brix/acid ratio of fruits. The *yft5* mutant accumulated lower levels of nutritional compounds than the knockout lines, yet its fruit size and weight remained largely comparable to those of the wild type.

Table 2. Nutritional quality analysis of ripe tomato fruits from different lines ($\mu\text{g}\cdot\text{g}^{-1}$ FW) *.

Plant Lines	Organic Acid		Flavonoids		Total Flavonoids
	Citric Acid	Chlorogenic Acid	Rutin	Chalconaringenin	
P86	264.28 $\pm$ 6.82 ^A	1.29 $\pm$ 0.14 ^{AB}	2.82 $\pm$ 0.55 ^C	0.42 $\pm$ 0.08 ^B	3.24 $\pm$ 0.62 ^C
<i>yft5</i>	252.57 $\pm$ 8.45 ^{AB}	0.82 $\pm$ 0.16 ^C	3.13 $\pm$ 0.86 ^C	0.19 $\pm$ 0.04 ^B	3.31 $\pm$ 0.87 ^C
<i>YFT5-KO11</i>	255.67 $\pm$ 15.56 ^{AB}	1.33 $\pm$ 0.11 ^A	4.76 $\pm$ 0.50 ^B	2.06 $\pm$ 0.54 ^A	6.82 $\pm$ 1.02 ^B
<i>YFT5-KO19</i>	231.78 $\pm$ 10.80 ^C	0.97 $\pm$ 0.06 ^{BC}	6.37 $\pm$ 0.23 ^A	2.77 $\pm$ 0.36 ^A	9.14 $\pm$ 0.26 ^A

* FW, fresh weight. Data are shown as means $\pm$ SD from three independent biological replicates, and were analyzed using one-way ANOVA followed by Duncan's test at $p < 0.01$. Different upper-case letters indicate statistically significant differences between groups.

3.3. The Change in Metabolites and ABA During Tangerine Tomato Fruit Development

To further explore the metabolites accumulation during fruit development in *Tangerine* tomatoes, the content of citric acid and phenolic compounds (flavonoids and chlorogenic acid) in fruits harvested at 38, 43 and 55 dpa was analyzed. Citric acid levels in the fruits of the *YFT5-KO* lines were consistently lower than those in the wild type throughout fruit ripening. Instead, they increased in *yft5* fruits at the mature green and breaker stage, but experienced a sharp decline at the ripe stage (Figure 2A). The accumulation of chlorogenic acid showed no significant change after the *SICRTISO* mutations, displaying a continuous decrease as the fruit ripens. However, at 38 dpa, its content in *YFT5-KO11* considerably surpassed that in all other lines (Figure 2B). Rutin kept a higher level in two knockout lines during the fruit development, while chalconaringenin started to accumulate after 38 dpa, and the levels in *YFT5-KO19* were significantly higher than those in other lines. The rutin and chalconaringenin levels in *yft5* showed no difference with wild type at all three stages (Figure 2C,D).

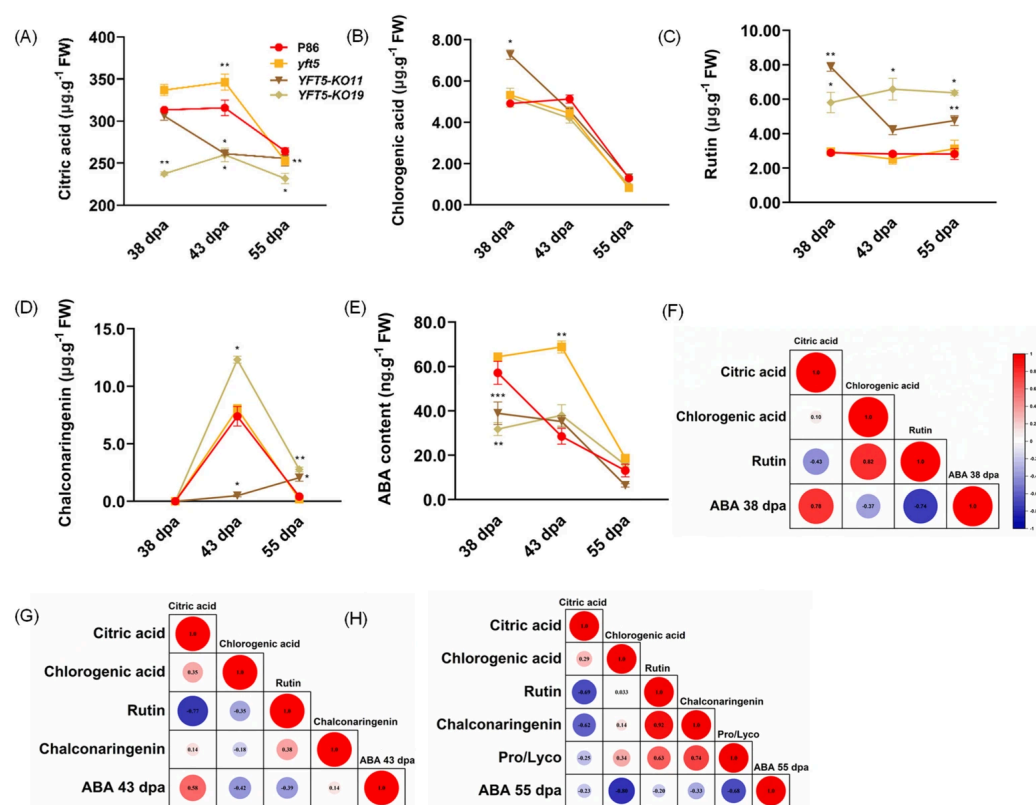


Figure 2. The metabolic changes in *tangerine* tomato fruits at different development stages. (A–E) The content of two organic acids, citric acid (A) and chlorogenic acid (B), two flavonoids, rutin (C) and chalconaringenin (D), and ABA levels (E) in P86, mutant *yft5* and YFT5-KO lines at 38, 43 and 55 dpa of fruit development. FW, fresh weight. Data are shown as means $\pm$ SD from three independent biological replicates. Asterisks indicate statistically significant difference calculated by the paired-samples *t*-test: *, $p \leq 0.05$. **, $p \leq 0.01$. ***, $p \leq 0.001$. (F–H) The Pearson correlation coefficient analysis of the correlation among nutritional components, organic acids, and ABA levels at 38 (F), 43 (G) and 55 dpa (H) of fruit development in four tomato lines at $p < 0.05$. The size of circles indicates the significance of correlation. The color scale indicates the strength of correlations. Pro/Lyco, concentration ratio of prolycopene to lycopene.

ABA was synthesized at the on-set of fruit ripening and was found to be deficient in the tomato *tangerine* mutant [5,16]. Therefore, ABA levels at three fruit development stages were detected. At 38 dpa, ABA levels were significantly reduced in the two knock-out lines but recovered to near-normal levels at subsequent stages. Unexpectedly, ABA levels in *yft5* were higher than other lines in all three stages, especially at 43 dpa (Figure 2E). Pearson correlation analysis revealed that the ABA level at three stages was found to be negatively correlated with rutin and chlorogenic acid content, and the ABA level at 55 dpa was negatively correlated with the content of all metabolites and especially significantly correlated with chlorogenic acid content and concentration ratio of prolycopene to lycopene. The prolycopene/lycopene ratio was positively correlated with chlorogenic acid and flavonoids at 55 dpa, and had negative correlation with citric acid levels at 55 dpa (Figure 2F–H).

3.4. The Transcriptomic Change Caused by *SlCRTISO* Mutations

To elucidate the transcriptomic basis underlying the phenotypic differences among *tangerine* tomatoes with distinct *SlCRTISO* alleles, total RNA was extracted from fruits of P86, *yft5*, and YFT5-KO19 at three developmental stages and subjected to RNA-sequenc-

ing. *YFT5-KO19* was selected as the representative knockout line for transcriptomic comparison because its metabolic profiles across development were more consistent with P86 and *yft5* than those of *YFT5-KO11* (Figure 2A–E). The PCA of the expression level (TPM) in nine samples revealed that the fruit transcriptome in *yft5* is more similar to P86 than *YFT5-KO19* in all fruit development stages (Figure 3A). Despite the distinct genetic backgrounds, these three lines clustered closely together on the PCA score plot (PC1 = 39.29%, PC2 = 18.56%) at 38 and 55 dpa, suggesting substantially similar transcriptomic profiles at the global expression level. However, the fruit transcriptomes showed the greatest variation across plant lines at 43 dpa. The 567 common differentially expressed genes (DEGs) shared by *YFT5-KO19* and *yft5*, as shown in the Venn diagram, were primarily enriched in three KEGG pathways: photosynthesis-antenna proteins, linoleic acid metabolism, and phenylpropanoid biosynthesis (Figure 3B,C). Notably, 1452 DEGs specifically found in *YFT5-KO19* were significantly enriched in the biosynthesis of unsaturated fatty acids and galactose metabolism, suggesting that primary metabolism may be more severely affected in *YFT5-KO19*. In contrast, no KEGG pathways were significantly enriched among the DEGs specifically detected in the *yft5* versus P86 comparison (Figure 3C).

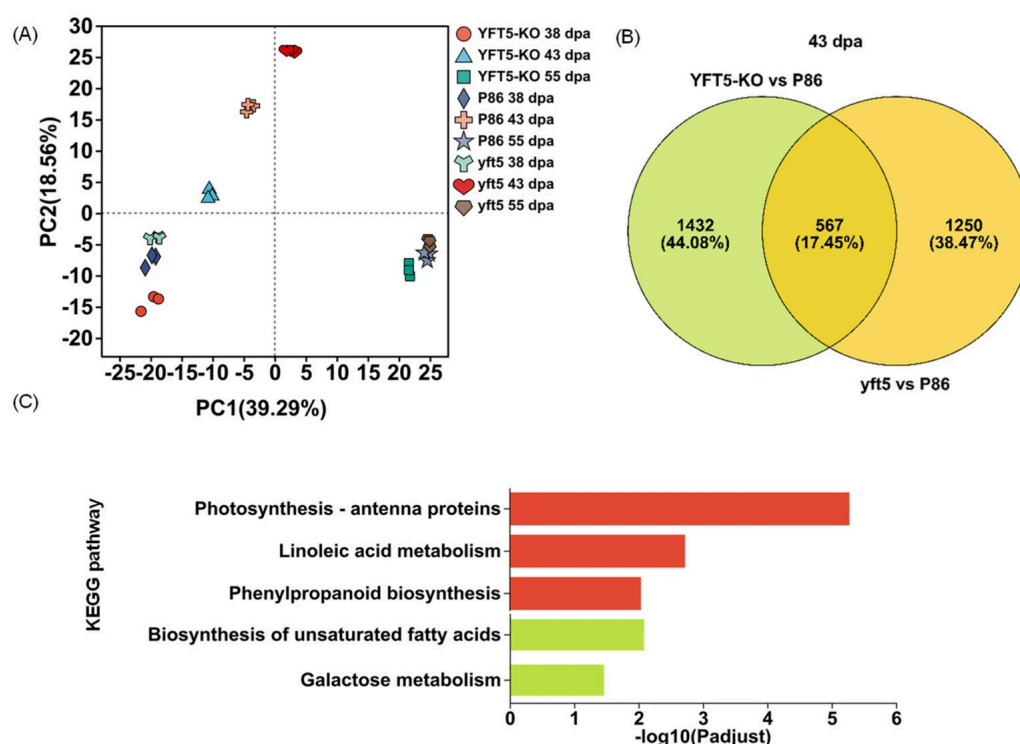


Figure 3. Transcriptomic differences between *yft5* and *YFT5-KO19*. **(A)** The principal component analysis (PCA) of all RNA-seq samples based on the TPM value. **(B)** The Venn diagram illustrating the distribution of different expressed genes (DEGs) in fruit samples of *yft5* and *YFT5-KO19* at 43 dpa. **(C)** The KEGG enrichment analysis of the shared DEGs between two lines (red) and specific DEGs in the *YFT5-KO19* versus P86 comparison (green). The listed pathways were enriched significantly at $P_{\text{adj}} < 0.05$. No pathways were enriched significantly in the *yft5* versus P86 comparison.

3.5. Impact of SICRTISO Mutations on Fruit Quality-Related Metabolic Pathways

The fruits of *YFT5-KO19* and *yft5* showed difference in terms of content of carotenoids, phenolic compounds (flavonoids and chlorogenic acid), citric acid, brix/acid ratio, and ABA levels. To further characterize the expression of genes involved in fruit quality-related metabolic pathways, we performed cluster analysis of DEGs identified

from the comparisons of *YFT5-KO19* and *yft5* versus the wild type at 43 dpa. The 3249 DEGs were grouped into six major subclusters, each exhibiting a distinct expression pattern (Figure 4). KEGG enrichment analysis was then conducted for the genes within each subcluster and key genes involved in tricarboxylic acid (TCA) cycle, phenylpropanoid-flavonoid biosynthesis, carotenoid biosynthesis, and starch and sucrose metabolism pathways were listed. Notably, all phenylpropanoid-flavonoid biosynthesis genes were confined to subclusters 1 and 2, which together accounted for over 70% of the total DEGs. These genes showed the lowest expression levels in *yft5* at 43 dpa. More specifically, *phenylalanine ammonia-lyase 2* (*PAL2*) and *chalcone synthase* (*CHS*) exhibited lower expression in *yft5* than in *YFT5-KO19* at 38 and 55 dpa, whereas *4-coumarate CoA ligase* (*4CL*) and *flavonoid 3'-hydroxylase* (*F3'H*) showed higher expression in *yft5* (Figure 5).

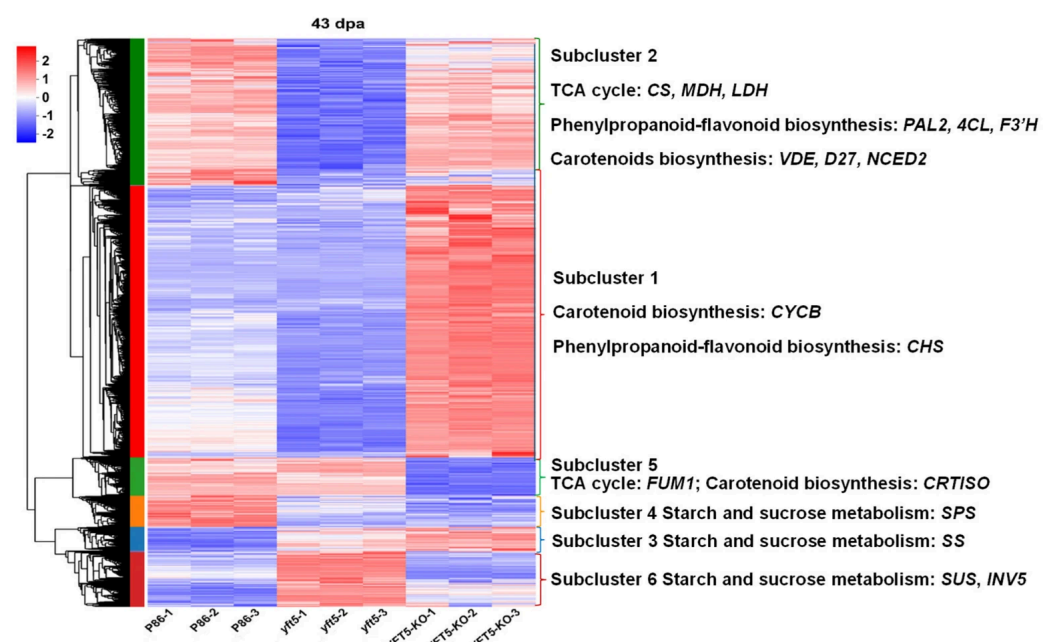


Figure 4. Cluster analysis of all DEGs based on $\log_{10}(\text{TPM})$ values in different tomato lines at 43 dpa. Three biological replicates were included for each line. Six major subclusters are shown in different colors. The color scale from blue to red represents gene expression levels from low to high. Key DEGs associated with the KEGG pathways enriched in each subcluster are listed on the right. TCA, tricarboxylic acid cycle. *CS*, citrate synthase; *MDH*, malate dehydrogenase; *LDH*, L-lactate dehydrogenase; *FUM1*, fumarate hydratase; *PAL*, phenylalanine ammonia lyase; *4CL*, 4-coumarate CoA ligase; *F3'H*, flavonoid 3'-monooxygenase; *CHS*, chalcone synthase; *SS*, starch synthase; *SPS*, sucrose phosphate synthase; *SUS*, sucrose synthase; *INV*, invertase; *CRTISO*, carotene isomerase; *CYCB*, chromoplast-specific lycopene β -cyclase; *VDE*, violaxanthin de-epoxidase; *NCED*, 9-cis-epoxycarotenoid dioxygenase; *D27*, DWARF27.

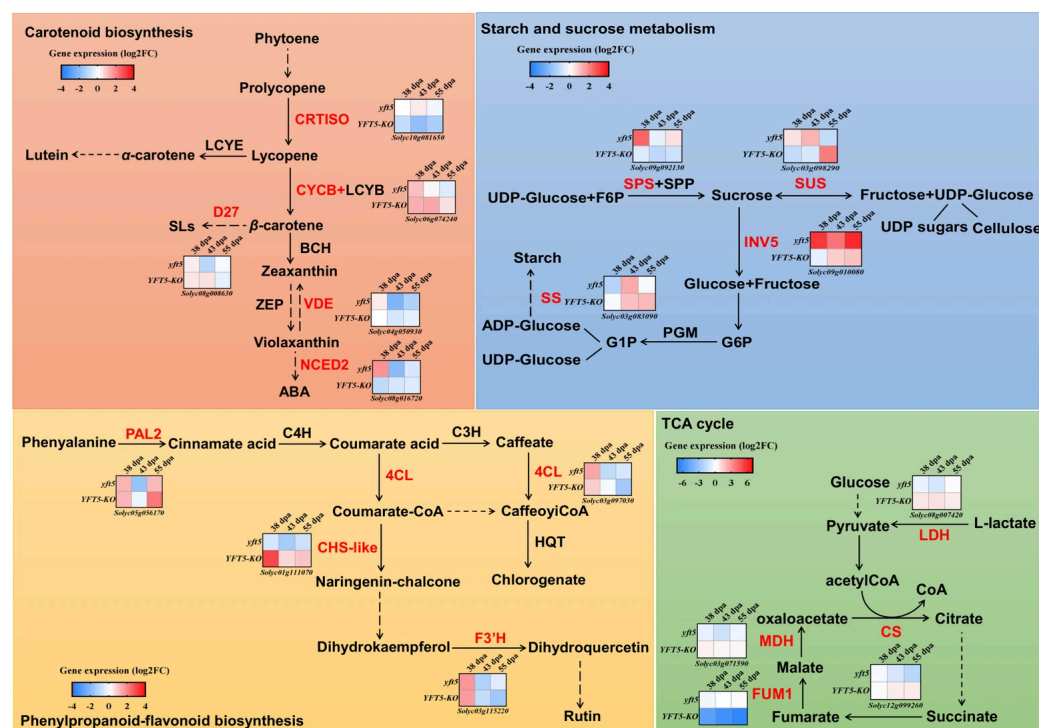


Figure 5. The gene expression difference between *YFT5-KO19* and *yft5* in four fruit quality-related metabolic pathways during fruit development. Diagrams of four metabolic pathways—carotenoid biosynthesis, starch and sucrose metabolism, phenylpropanoid–flavonoid biosynthesis, and the TCA cycle—are highlighted with red, blue, yellow, and green backgrounds, respectively. The expression levels of DEGs in these pathways are displayed as heatmaps, with gene names labeled in red. The color scale represents log₂ fold change (log₂FC) values relative to the wild type: blue indicates downregulation, white indicates no change, and red indicates upregulation. Solid arrows denote single-step reactions, dashed arrows denote multi-step reactions, and double-headed arrows indicate reversible reactions. LYCE, lycopene ε-cyclase; LCYB, lycopene β-cyclase; BCH, β-carotene hydroxylase; ZEP, zeaxanthin epoxidase; SPP, sucrose phosphate phosphatase; PGM, phosphoglucomutase; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; C4H, cinnamate-4-hydroxylase; C3H, coumarate 3-hydroxylase; HQT, hydroxycinnamoyl-Co A quinate hydroxycinnamoyl transferase.

In addition to phenylpropanoid-flavonoid genes, subclusters 1 and 2 also contained most of the downstream carotenoid biosynthesis genes and TCA cycle genes. The gene *chromoplast-specific lycopene β-cyclase* (*CYCB*) exhibited lower expression level in *yft5* than in *YFT5-KO19* throughout the fruit ripening, whereas the expression level of violaxanthin de-epoxidase (*VDE*) and 9-*cis*-epoxycarotenoid dioxygenase 2 (*NCED2*) was upregulated in *yft5* at 38 dpa, followed by downregulated expression at later stages (Figures 4, 5 and A1). The *DWARF27* (*D27*) showed lower expression in *yft5* at early stages but slightly surpassed that in *YFT5-KO19* at 55 dpa. For TCA cycle genes, the expression levels of *citrate synthase* (*CS*), *malate dehydrogenase* (*MDH*) and *lactate dehydrogenase* (*LDH*) in subcluster 2 are lower in *yft5* than those in *YFT5-KO19* at all development stages. However, another TCA cycle gene *fumarate hydratase* (*FUM1*) in subcluster 5 showed a completely opposite expression pattern, being significantly downregulated in *YFT5-KO19* during fruit development while remaining unchanged in *yft5* compared with P86. This pattern is consistent with the expression profile of the target gene, *SICRTISO*, in this study (Figures 4 and 5).

Genes involved in starch and sucrose metabolism were distributed across subclus-

ters 3, 4, and 6, reflecting the diverse expression patterns of these genes among the different lines. *Invertase 5 (INV5)* was upregulated to higher levels in *yft5* at all three developmental stages, whereas *sucrose synthase (SUS)* showed elevated expression in *yft5* only at the first two stages and was instead significantly induced in *YFT5-KO19* at 55 dpa. *Sucrose phosphate synthase (SPS)* exhibited higher expression levels in *yft5* than in *YFT5-KO19* at all three stages, with a particularly significant upregulation at 38 dpa. In contrast, the expression of *starch synthase (SS)* fluctuated throughout fruit ripening and displayed no consistent pattern in different plant lines (Figures 4 and 5).

4. Discussion

This study evaluated the effect of different *SICRTISO* mutations on agronomic traits and nutritional quality of *tangerine* tomatoes. *Tangerine* tomatoes are known to over-accumulate 7'9'7'9'-tetra-cis-lycopene (prolycopene) in fruits due to the damaged conversion from prolycopene to lycopene [53,54]. The knockout and T2852C point mutation of *SICRTISO* caused varying degrees of protein lesion, leading to different accumulation of prolycopene (Figure 1), which is consistent with the *SICRTISO* mutants *tangerine*³¹⁸³ and *tangerine*^{mic}, characterized previously [29]. The mutant *tangerine*³¹⁸³ contains a deletion in the promoter region, leading to impaired *SICRTISO* expression, whereas the *SICRTISO* protein synthesis is aborted in *tangerine*^{mic} due to a deletion in the first exon, thus containing higher percentage of prolycopene and barely any lycopene. Three other *tangerine* mutants carrying point mutations in *SICRTISO* also contain various amount of prolycopene and lycopene [30]. Therefore, the prolycopene/lycopene ratio depends on the severity of the mutation.

The high accessibility to human body is a huge advantage for *tangerine* tomatoes due to the higher bioavailability of prolycopene [26,28]. Apart from this, other fruit quality traits in *tangerine* tomatoes were also studied in this work. The brix/acid ratio of fruits in two knockout lines and mutant *yft5* were all improved, owing to the decreased titratable acidity and increased brix (Table 1). The specific citric acid levels measured by UPLC also showed significant reduction in those *tangerine* tomatoes (Table 2). The previous investigation of fruit flavor in *tangerine*³¹⁸³ revealed the alteration of volatile aroma, and the content of sugars and acids tends to be season-dependent. The brix and acid indexes measured from fruits harvested in June were consistent with our work [32]. Research on the agronomic traits in *CRTISO* mutants revealed the reduced fruit yield in the *tangerine*^{mic} mutant, as well as the shorter panicles and fewer grains in the rice mutant *dn* [16,22]. In this study, the fruit size and weight were largely reduced in the *YFT5-KO* lines but not significantly affected in *yft5* (Table 1), which reflected the advantage of *yft5* in preserving the fruit flavor and physical traits.

Phenolic compounds are important contributors to the nutritional quality of tomato fruits. The content of total flavonoids and individual flavonoid were dramatically higher in two knockout lines throughout the fruit development except for the chalconaringenin content in *YFT5-KO11* at 43 dpa (Table 2 and Figure 2C,D). The alteration of flavonoid biosynthesis in petals has been reported in *BnaCRTISO* knockout mutant in *Brassica napus* L., but the naringenin chalcone content was significantly decreased, together with the increased lycopene [55]. It might be attributed to the positive association of the prolycopene/lycopene ratio and flavonoid levels, as illustrated in this work (Figure 2H). Research in rice and tomato have shown that *CRTISO* mutations cause the reduction in ABA level [16,17,22,56]. Consistent with the earlier observation, ABA levels in two knockout lines were low at 38 dpa and recovered to normal levels thereafter, whereas *yft5* exhibited the highest ABA level among all lines at 43 dpa (Figure 2E). Complex carotenoids flux readjustment might occur during the fruit development in response to

the alteration caused by *SICRTISO* mutations that leads to the alteration of downstream ABA level. ABA was confirmed to influence pigment accumulation in both climacteric and non-climacteric fruits [10,57,58]. Several studies in tomatoes have demonstrated that reduced endogenous ABA is associated with deeper-colored fruits accumulating more carotenoids and flavonoids [8,12,59]. Consistently, our study observed a negative correlation between ABA levels and the content of flavonoids (Figure 2F–H).

The point mutation and loss-of-function mutation of *SICRTISO* both induced significant transcriptomic changes in phenylpropanoid biosynthesis pathway, which are in line with the change in phenolic compounds levels in *YFT5-KO* lines and *yft5* (Figure 3 B,C). Furthermore, the expression of photosynthesis-antenna genes was also affected, consistent with the transcriptomic change reported in melo (*Cucumis melo*) *CmCRTISO* mutant *yofi* [60], and the reduced photosynthesis rate has been demonstrated in rice *OsCRTISO* mutants *zabra2* and *pgl13* [21,23]. Interestingly, the galactose metabolism and unsaturated fatty acid biosynthesis pathways were uniquely enriched in *YFT5-KO19* fruits. Galactose is a key component of pectic polysaccharides in the primary cell wall and plays essential roles in cell wall biosynthesis, remodeling, and cell expansion during fruit development [61,62]. In tomato, altered galactose metabolism has been implicated in changes in fruit firmness through its effects on cell wall pectin composition [63], suggesting that this pathway may influence fruit texture. Unsaturated fatty acids are critical for membrane biogenesis and fluidity, processes also required for cell enlargement [64]. These observations raise the possibility that transcriptional changes in these pathways are linked to the reduced fruit size and weight in the *YFT5-KO* lines (Table 1), although this proposed connection remains to be validated by direct biochemical or histological evidence.

The DEGs involved in four metabolites pathway at 43 dpa were studied. In each pathway, the expression levels of key genes determining the final fruit trait indexes were significantly altered (Figure 5). For ABA biosynthesis, previous studies have proved that *NCED2* is not the key gene in ABA metabolism in tomato fruits [65], but silencing of *SINCED2* leads to a decrease in ABA level [66]. Thus, the significantly upregulated *NCED2* expression in *yft5* at 38 dpa might be the reason for the high ABA level (Figure 2 E, Figures 5 and A1). The gene *CHS* and *CS* directly determines the content of chalcone-naringenin and citric acid, respectively [67,68], and they were both highly expressed in *YFT5-KO19*, but had relatively lower expression in *yft5*. This pattern aligns with the chalcone-naringenin data but contrasts with the reduced citric acid levels in *YFT5-KO19*. This discrepancy may result from limited substrate availability due to *FUM1* downregulation and impaired fatty acid metabolism, which could restrict the supply of oxaloacetate and acetyl-CoA for citrate synthesis (Figures 2 and 5). Additionally, citrate is actively consumed through the TCA cycle and gluconeogenesis during tomato fruit ripening, where organic acids are converted into soluble sugars [69,70]. Enhanced carbon flux through this pathway could deplete citrate pools in *YFT5-KO19*, contributing to the lower citric acid content and higher brix value in fruits (Tables 1 and 2). Regarding *INV5* as the core gene for fruit sugar accumulation [71], its expression was higher in *yft5*, thus leading to high brix/acid ratio in *yft5* fruits (Table 1 and Figure 5).

Despite the insights gained, several limitations of this study should be acknowledged. It should be noted that transcriptomic analysis was conducted on only one knockout line (*YFT5-KO19*). This line was selected because its metabolic profiles across development were more consistent with P86 and *yft5* than those of *YFT5-KO11*, which showed atypical reductions at 43 dpa. Although both knockout lines carry functionally equivalent null alleles, we cannot exclude the possibility that some transcriptomic changes are line-specific. Additionally, the RNA-seq results for certain pathways, partic-

ularly those related to sugar and organic acid metabolism, were not independently validated by qRT-PCR. Future studies incorporating both knockout lines and comprehensive qPCR validation would further strengthen these findings. In addition, further studies should analyze the metabolites change more comprehensively, especially in terms of tomato flavor. Alterations of specific sugars and acids in fruits should be analyzed by gas chromatography-mass spectrometry (GC-MS), and the volatile aroma compounds should also be measured. Additionally, the *tangerine* tomatoes used in this study are in the background of the P86 cultivar. More *tangerine* tomatoes carrying new *SICRTISO* alleles or in other genetic backgrounds should be characterized, so as to give a more convincing and informative evaluation of the fruit quality of *tangerine* tomatoes.

5. Conclusions

This study demonstrated that the two *tangerine* tomato mutants exhibited a higher brix/acid ratio than the wild type, regardless of mutation severity. Although the *tangerine* tomato carrying the T2852C point mutation (*yft5*) accumulated less prolycopene than the *SICRTISO* knockout lines (*YFT5-KO11* and *YFT5-KO19*), its fruit size and weight remained comparable to those of the wild type without significant reduction. This phenotypic advantage may be attributed to the less significant perturbation of primary metabolism in *yft5*, particularly in pathways related to cell expansion, such as unsaturated fatty acid biosynthesis and galactose metabolism. Regarding fruit nutritional quality, the *YFT5-KO* lines accumulated higher levels of rutin and chalconaringenin throughout the fruit development, whereas the phenolic compounds in *yft5* showed no significant changes compared with the wild type. ABA levels across the three developmental stages exhibited strong negative correlations with pigment content, including flavonoids and the prolycopene-to-lycopene ratio. These metabolic changes were consistently supported by transcriptomic alterations in four key metabolic pathways, particularly at 43 dpa, when gene expression diverged most markedly among the wild type, *yft5*, and *YFT5-KO19*. For breeding purposes, the *SICRTISO*^{W382R} allele is particularly suitable when agronomic performance and fruit size are prioritized, whereas the *SICRTISO*^{KO} alleles are advantageous for enhancing nutritional quality through elevated phenolic compounds. Future studies integrating these alleles into diverse genetic backgrounds will help optimize the balance between nutritional value and horticultural performance in *tangerine* tomato varieties.

Author Contributions: Conceptualization, A.X.; methodology, A.X.; software, A.X.; formal analysis, A.X.; investigation, A.X.; data curation, A.X.; writing—original draft preparation, A.X.; writing—review and editing, A.X.; supervision, L.Z.; project administration, L.Z.; funding acquisition, L.Z. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The RNA-seq raw data reported in this paper has been deposited in the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA1483294 and are publicly available at <https://www.ncbi.nlm.nih.gov/sra/PRJNA1483294> (accessed on 5 August 2026). Other data supporting the findings of this study are included in the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

UPLC-MS	Ultra performance liquid chromatography–mass spectrometry
UHPLC	Ultra-high performance liquid chromatography
FW	Fresh weight
PCA	Principal component analysis
KEGG	Kyoto encyclopedia of genes and genomes
DEG	Differentially expressed genes

Appendix A

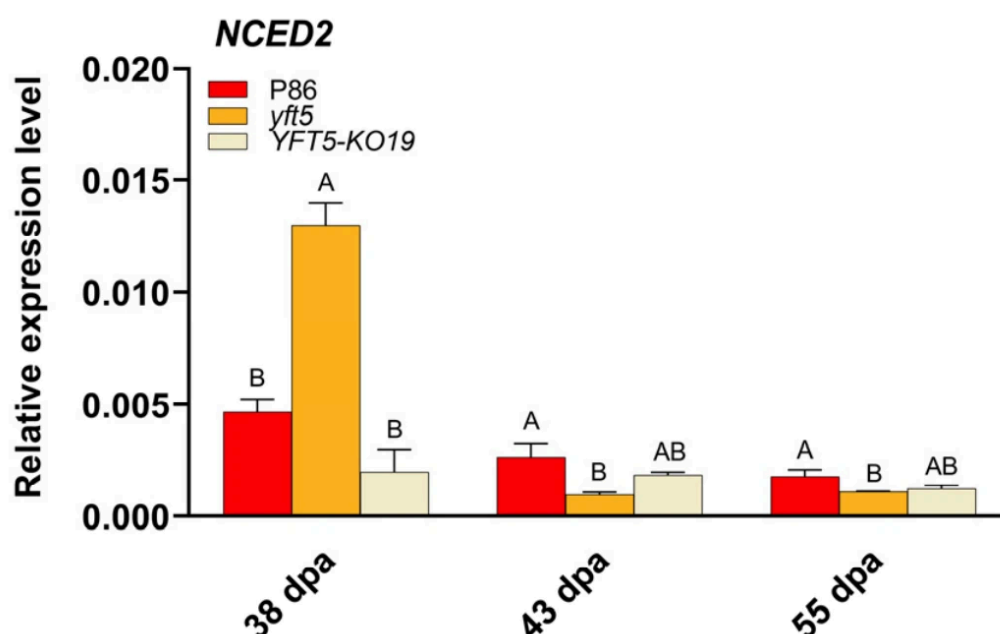


Figure A1. RT-qPCR results of *NCED2* in tomato fruits at 38, 43 and 55 dpa from P86, *yft5* and *YFT5-KO19* using *ACTIN* (*Solyc03g078400*) as an internal control. Data are shown as means $\pm$ SD from three independent biological replicates, and were analyzed using one-way ANOVA followed by Duncan's test at $p < 0.01$. Different uppercase letters indicate statistically significant differences between groups.

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