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Article

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Transcriptomic and metabolic analysis uncovers the role of light quality in carotenoid accumulation of grapefruit during ripening

Running Head: Light changes carotenoid accumulation in grapefruit

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Abstract

Light, a crucial environmental signal, is involved in the regulation of secondary metabolites. To understand the mechanism by which light influences carotenoid metabolism, grapefruits were bagged with four types of light-transmitting bags that altered the transmission of solar light. We showed that light-transmitting bagging induced changes in carotenoid metabolism during fruit ripening. Compared with natural light, red light (RL)-transmittance treatments significantly increased the total carotenoid content by 142%. Based on weighted gene co-expression network analysis (WGCNA), ‘red’, ‘darkred’, ‘yellow’, ‘brown’ and ‘midnightblue’ modules were remarkably associated with carotenoid metabolism under different light treatment. Transcriptome analysis identified the transcription factors (TFs) bHLH74/91/122, NAC56/78/90/100, MYB/MYB308, WRKY7/55, MADS29/AGL61, ERF043/118 as being involved in the regulation of carotenoid metabolism in response to RL. Under RL treatment, these TFs regulated the accumulation of carotenoids by directly modulating the expression of carotenogenic genes, including \textit{PSY}, \textit{Z-ISO2}, \textit{ZDS6}, \textit{LCYB}, \textit{LCYE}, \textit{CHYB}, \textit{CCD1-1/1-3}, \textit{CCD4-2} and \textit{NCED2/3}. Based on these results, a network of the regulation of carotenoid metabolism by light in citrus fruits was preliminarily proposed. These results showed that RL treatments have great potential to improve coloration and nutritional quality of citrus fruits.

Keywords: grapefruit, light, bagging, carotenoid, RNA-sequencing, WGCNA.
Introduction

Carotenoids are a large class of natural lipid-soluble pigments that are extensively distributed in plants and play important roles in plant growth and development, being involved in photosynthesis, photomorphogenesis, photoprotection, and phytohormone synthesis\(^1,2\). The accumulation of carotenoids confers on many fruits and vegetables their various colors, such as yellow, orange, and red hues\(^3\). In addition, their catabolites provide precursors for the synthesis of abscisic acid (ABA) and strigolactones (SLs), which participate in various biological processes and stress responses\(^4\). In humans, carotenoids in plant-based foods are an important source of dietary vitamin A, which is essential for health and nutrition, and carotenoid-rich diets are correlated with a significant reduction in the risks of chronic diseases such as cancers, cardiovascular diseases, and several degenerative diseases\(^2,5\). The concentrations of carotenoids in fleshy fruits thus greatly influence their commercial and nutritional value.

The grapefruit (\textit{Citrus paradisi} Macf.) is an economically important tropical cultivated citrus fruit\(^6\). In 2018, the planted area of grapefruit in China was about 9200 hectares, about 25% of the global planted area, while annual production (around 5 million tons) accounted for approximately 54% of global output, indicating that grapefruit is an important part of China’s citrus production (FAO statistics, http://www.fao.org/home/en/). Red grapefruit is becoming more and more preferred by consumers for its unique flavor and attractive pulp color. Besides having an abundance of a wide variety of health-promoting compounds such as flavonoids, dietary fiber, and vitamin C\(^7\), grapefruits are richer in carotenoids than other citrus species and thus...
represent an ideal material for investigating carotenoid metabolism.

The pathway of carotenoid biosynthesis has been clearly established in plants. The five-carbon prenyl diphosphate isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP) are synthesized in plastids via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The subsequent condensation of two molecules of geranylgeranyl diphosphate (GGPP), produced from IPP and DMAPP, by phytoene synthase (PSY) generates the colorless 15-cis-phytoene. After sequential desaturation and isomerization reactions catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), and ζ-carotene isomerase (Z-ISO) and ζ-carotenoid isomerase (CRTISO), respectively, phytoene is converted into the red all-trans-lycopene. The production of α- and β-carotene from lycopene involves a set of cyclization reactions catalyzed by lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) or LCYB alone, representing the β, ε- and β, β-branches of the pathway, respectively, and this step is the pivotal branch point in carotenoid metabolism. Next, α-carotene is converted into lutein by β-ring hydroxylase (CYP97A) and ε-ring hydroxylase (CYP97C) of the cytochrome P450 family. The production of zeaxanthin from β-carotene is catalyzed by β-carotene hydroxylase (CHYB), and violaxanthin is generated via antheraxanthin by zeaxanthin epoxidase (ZEP). The cleavage of carotenoids is catalyzed by the proteins of carotenoid-cleavage genes (CCD or NCED), producing apocarotenoids such as β-ionone, β-citraurin, and ABA. Carotenoid biosynthesis and degradation are coordinated by a range of enzymes encoded by structure genes and transcription factors (TFs). These structure genes have
been identified and isolated in many plant species to date. However, only a few transcription factors related to carotenoid metabolism have been identified in plants, including RIPENING INHIBITOR (RIN) and FRUITFULL1/2 (FUL1/2) in the MADS-box family; PIF1, TOMATO AGAMOUS LIKE1 (TAGL1), SIMADS1, SINAC1/4, SIAP2a, SIERF6, and SIBBX20 in tomatoes; CsMADS5/6 and CrMYB68 in oranges (flavedo); CpEIN3a, CpNAC1/2, and CpbHLH1/2 in papaya; AdMYB7 in kiwifruits; and R2R3-MYB subgroup Reduced Carotenoid Pigmentation 1/2 (RCP1/2) in monkey flower species. Compared with that of anthocyanin metabolism, the transcriptional regulation of carotenoid metabolism is far from understood.

Light not only provides the energy required for photosynthesis but also participates in the regulation of a variety of metabolic processes as the crucial medium for the exchange of information between the plant and external environment during development. An increasing number of investigations suggest that light signals also play a fundamental role in secondary metabolism in fruit. However, the majority of studies have focused on the effect of postharvest light treatment on fruit quality, with only a few referring to the impact of developmental light treatment on fruit quality. As an effective method of protecting fruit from insect infestations, bird attack, and sunburn as well as reducing disease incidence rate and chemical residues, fruit bagging is extensively used in modern orchards. Light-transmitting paper bags of different colors can absorb the light waves of the corresponding colors, making their use a feasible approach for investigation of how light influences phytochemicals metabolism.
This study was carried out to know the role of light quality on the carotenoid accumulation in grapefruit and understand the transcriptional regulatory mechanism underlying light signals during fruit ripening. The carotenoid level of ‘Huoyan’ grapefruit pulp treated with different light-transmittance during the ripening were compared, WGCNA were employed to identify the key genes and TFs responsible for carotenoid metabolism during the process. Based these results, a regulatory network of carotenoid metabolism in response to red-light was preliminarily proposed. These findings provide new insight into carotenoid metabolism and demonstrate a potential approach for improvement of the coloration and nutritional quality of citrus fruit and other horticultural crops.

Results

Effects of light transmittance on TSS, TA, and CCI during fruit ripening

Compared with that in the dark shade treatment (DS), the TSS content of the grapefruits treated with RL, BL, and WL gradually increased during fruit ripening and were significantly higher than that in DS treatment at 215 DAB ($p < 0.05$) (Fig. 1A). The effect of light treatment on TA content is different (Fig. 1B). It is worth noting that the TA content under RL and WL treatment slightly rose during fruit ripening while the TA content gradually decreased in BL and DS treatment. CCI shared similar trends and were gradually increased in all light treatments (Fig. 1C).
Effects of light transmittance on carotenoid accumulation during fruit ripening

Five major carotenoids were identified from ‘Huoyan’ grapefruit pulp, including β-carotene, phytofluene, ζ-carotene, lycopene, and 9-cis-violaxanthin (Table S1). The carotenoid profiles differed in the four light treatments, and the ‘Huoyan’ grapefruit pulp was rich in β-carotene and lycopene (Fig. 2). Compared with the control group (DS), the total carotenoid content was the highest in the grapefruits treated with RL (2.4-fold), followed by the grapefruits treated with BL (1.69-fold) and WL (1.59-fold) at 215 DAB. Subsequently, we found that the content of β-carotene, ζ-carotene lycopene and phytofluene gradually increased as fruit ripening under different light treatments while 9-cis-violaxanthin content didn’t show marked changes. Compared with DS, the β-carotene and lycopene content significantly increased under RL treatment and remarkably higher than those in BL and WL treatment at 215 DAB.

Transcriptome profiles during fruit ripening

Average clean reads number of mRNA libraries for eight samples ranged from 20.98 to 23.09 million (Table S2). The alignment of the clean reads against the reference genome and reference gene sequences generated a total of 25,694 unigenes (Table S3). In four light treatments, the median of gene expression level ranged from 0.76 to 0.88 and there were differences between the samples (Fig. S2). The fruit samples in BL treatment showed the lowest median, 0.76, at 215 DAB, while the samples treated with WL presented the biggest median value of 0.88 at 185 DAB. However, in the DS and RL treatment groups, the gene expression levels for simples were relatively stable.
during ripening.

Identification of differentially expressed genes (DEGs)

Based on RNA sequencing results, a total of 5541 DEGs were identified during fruit ripening, with 2305, 1381 and 1855 DEGs showing differential expression between DS and RL, DS and BL, and DS and WL, respectively (Fig. 3A). In the RL group, 1817 and 488 DEGs were identified at 185 and 215 DAB, respectively, and the number of DEGs was significantly higher than other light treatments. The numbers of extremely significant DEGs throughout fruit ripening were 152, 167 and 173, respectively, after RL, BL, and WL treatment (Fig. 3B). Notably, at the two indicated detection points, the unique DEGs of RL, BL and WL treatment, respectively, reached a maximum of 1650 at 185 DAB, 598 DEGs at 215 DAB and 1159 DEGs at 185 DAB.

Weighted gene co-expression network analysis (WGCNA)

The WGCNA was performed using 12619 unigenes (FPKM > 1, the top 50% of variance), which were classified into twenty-four modules (Fig. 4), of which the ‘red’, ‘darkred’, ‘yellow’, ‘brown’ and ‘midnightblue modules were remarkably associated with carotenoid metabolism under different light condition during fruit ripening ($p < 0.05$). The analysis of the correlation between gene expression and carotenoid accumulation demonstrated that the ‘red’ and ‘darkred’ module, respectively, contained 469 (3.7%) and 53 (0.4%) genes and was significantly positively correlated with the content of carotenoid. Besides, ‘yellow’ module was positively associated with
phytofluence content ($r=0.78$, $p=0.02$). However, ‘brown’ and ‘midnightblue’ modules were highly negatively related with phytofluence content, which contained 1882 genes (14.9%), with a correlation coefficient of $-0.75$ ($p = 0.03$) and $-0.72$ ($p = 0.04$), respectively. These results indicate that genes in these modules were potentially correlated with carotenoid accumulation under different light conditions.

Expression analysis of genes related to carotenoid metabolism

In the carotenoid metabolic pathway (Fig. 5a), a total of eight structural genes, including $GGPPS1$, $ZEP1/2$, $CCD1-1/1-3$, $CCD4-1$, $NCED2/3$, were differentially expressed in response to RL during ripening (Table S4). RL significantly induced the transcription for carotenoid biosynthetic and cleavage genes during grapefruit ripening (Fig. 5A). Among the 3080 genes in the carotenoid co-expression modules, a total of 235 transcription factors (TFs), which were enriched in 52 gene families such as ERF (21), bHLH (19), MYB (18), WRKY (15), NAC (9) and MADS (7), were identified (Fig. S3). In order to further excavate the transcription factors responding to RL, we screened the differentially expressed transcription factors between RL and DS group. In order to further excavate the transcription factors responding to RL, we screened the differentially expressed transcription factors between RL and DS group. Subsequently, a total of forty-eight differentially expressed TFs in response to RL, including ERF (13), WRKY (7), bHLH (5), NAC (4), C3H (4), bZIP (4), MYB (3), GRAS (3), HSF (2), Dof (2), MADS (2) and GRF (1), were identified as candidate TFs modulating carotenoid biosynthesis in response to RL during fruit ripening (Fig. 5B, Table S5).
Visualization of gene networks

In order to identify the hub gene underlying carotenoid metabolism under different light-transmittance conditions, the co-expression for structural genes and regulators was visualized using Cytoscape. In carotenoid co-expression modules, forty TF members—derived from the ERF (10), WRKY (9), bHLH (5), bZIP (4), NAC (3), MYB (3), C3H (2) Dof (2), GRAS (1) and GRF (1) families—were identified as the key genes related to carotenoid metabolism. Meanwhile, twelve structural genes, namely *IPI*, *PDS*, *ZDS4*/*ZDS7*, *CRTISO1/3*, *CYP97C1/2*, *CCD1-2*, *CCD4-2*, *CCS*, *NXS*, which directly involved in the carotenoid biosynthesis and cleavage, were identified as the key regulatory genes in co-expression network (Fig. 6). In addition, we found that thirty-two TFs, including ERF, WRKY, bHLH, NAC and MYB family members were co-expressed with carotenoid cleavage gene *CCD4-2* and six TFs had a co-expression relationship with *CYP97C1* (Table S6). Notably, there was a co-expression relationship between MYB4 and *CCD1-2*, *CCS*, *PDS*. These results suggested that above transcription factors and structural genes might interact with each other to regulate the flux for carotenoid in grapefruits.

Discussion

Light signals play a vital role in carotenoid metabolism. Although many studies have investigated the effects of postharvest light treatments, such as LED, pulse, and ultraviolet light, on carotenoid metabolism, the role of light quality in carotenoid
metabolism underlying fruit ripening previously had not been elucidated. In this work, different light-transmittance bagging treatments were used to understand the influence of light quality on grapefruits throughout the ripening process at the metabolic and molecular levels.

Existing research indicates light irradiation modulates the biosynthesis and catabolism of carotenoids in fruit and modifies the concentration and composition of carotenoids. The total carotenoid content was reduced upon ripening in covered tomatoes and peppers. In grapefruit peel, an unusual pattern of lycopene accumulation in might be associated with the developmentally regulated differentiation of chromoplasts mediated by light. Light deprivation promoted peel degreening and reduced carotenoid accumulation in mandarins and sweet orange fruits. In contrast, light irradiation enhanced carotenoid accumulation and external quality during mandarin fruit development. Here, our results showed that carotenoid (total carotenoid, β-carotene, phytofluene, and lycopene) accumulation was significantly induced by red-transmittance bagging treatments (Fig. 2), suggesting that RL played significant positive role in carotenoid accumulation in grapefruit pulp. These are evidence of the promotion of carotenoid metabolism by light in fruit.

Lighting factors can regulate secondary metabolism by light quality, light intensity, and light irradiation time in plant. Red LED light (660 nm) activated the expression of VvNCED1 in ripening grape skin (Vitis vinifera L.), but had no effect on the carotenoid content of citrus juice sacs. Blue LED light (470 nm) treatment stimulated carotenoid accumulation by upregulating the expression of the CitPSY gene in the juice
sacs of three citrus varieties (mandarin (*Citrus unshiu* Marc.), Valencia orange (*C. sinensis* Osbeck) and Lisbon lemon (*C. limon* Burm.f.))\(^\text{32}\). In the present study, we found that RL promoted lycopene and β-carotene accumulation along with upregulation of *PSY*, *Z-ISO1/2*, *ZDS1/6*, *LCYB*, *LCYE*, *NCED3* and *CCD1-3* (**Fig. 2 and 5**), which was similar with the upregulation of the *PSY* gene induced by continuous red light in *Arabidopsis thaliana* seedlings, leading to increases in carotenoid content\(^\text{33}\). However, blue- and white-light transmittance treatment have no significant effect on carotenoid content during grapefruit ripening. These results revealed that the regulatory roles of light in carotenoid metabolism also depends on the light quality.

A large number of TFs have been reported to be involved in carotenoid metabolism via transcriptional regulation of key structural genes in plant. Here, multiple members of ERF, NAC, WRKY, MYB, MADS, bHLH families be identified as hub genes for modulating carotenoid flux (**Fig. 5 and 6**). Work in *Arabidopsis thaliana* revealed that the PIF1 (phytochrome interacting factor 1) transcription factor suppressed *PSY* transcript by directly binding the G-box motifs and further regulated carotenoid metabolism\(^\text{34}\). Zhou et al. showed that CpbHLH1/2 promoted lycopene degradation to carotenoids by upregulating the transcripts of the lycopene β-cyclase genes (*CpLCYB* and *CpCHYB*) in response to strong light during papaya ripening\(^\text{18}\). Blue- and red-light supplementation irradiation to tomato fruits at anthesis facilitated lycopene biosynthesis, which was considered to be related with regulation of the photoreceptor HY5 (ELONGATED HYPOCOTYL5) and PIFs upon the expression of *PSY1*\(^\text{35}\). Here, in RL-treated grapefruit, bHLH62/74/91/122 in ‘brown’ module negatively correlated with
phytofluene presented markedly down-regulated trend coupled with transcript increase of *PSY* and CHYB ([Fig. 2, 5 and 7](#)), which accounted for higher phytofluene level, and these results are paralleled with carotenoid increase of content in SlPIF4-silenced tomato\(^\text{36}\).

Some ripening related regulators have been shown the regulatory role in carotenoid metabolism. In tomato, MADS-box TFs RIN gene was reported to specifically regulating accumulation of lycopene by positively regulated carotenoid biosynthetic genes (including *PSY, Z-ISO, CRTISO*) and negatively regulated carotenoid downstream genes *LCYB* and *LCYE*, while FUL homologs FUL1/2 regulated overall carotenoid pathway by targeted multiple carotenogenic genes\(^\text{37,38}\). In sweet orange (*Citrus sinensis*), CsMADS5/6 activated expression for carotenogenic genes, including *PSY, PDS, LCYb1/CCD1* via directly binding its promoter and thus modulated carotenoid metabolism\(^\text{13,14}\). In ‘darkred’ module, we found that up-regulation of MADS29 in response to RL was accompanied by the increased expression level of *Z-ISO2, LCYB* as well as *CCD1-3*, which facilitated carotenoid accumulation ([Fig. 2, 5 and 7](#)). By contrast, the significantly reduced expression of AGL61 for ‘darkred’ module during ripening suggested their negative correlation with phytofluene accumulation in grapefruit ([Fig. 5 and 7](#)). Recently, PpERF3 has been shown to be involved in ABA biosynthesis by activating *PpNCED2/3* transcription during peach fruit ripening\(^\text{39}\). Here, PpERF3 homolog ERF012 was down-regulated in response to RL, which suggested ERF012 were highly likely to be involved in carotenoid process mediated by RL. On the contrary, RL remarkably promoted transcript of ERF043/118
and NCED2/3 shared similar expression patterns with them (Fig.5 and 7). Above analysis indicated ERF TFs differently respond to RL and collaboratively regulated carotenoid accumulation. In the Arabidopsis, suppression of AtRAP2.2 leads to reduction of PSY and PDS transcript\(^{40}\). In rice leaves, AP2/ERF genes were negatively associated with carotenoid accumulation under both blue- and red-light treatments\(^{41}\). Here, we also found multiple ERF TFs (ERF023-like/025/026) in ‘brown’ module displayed negative correlation with phytofluence accumulation in response to RL.

Another fruit ripening related TF NACs were also reported to be involved in carotenoid metabolism. In tomato SINAC4/19/48 RNAi fruit, the transcript levels of PSY were reduced and thus resulted in decreased lycopene\(^{42,43}\). However, the overexpression of SINAC1 reduced lycopene content, which was associated with a reduction in SIPSY and an increase in SILCYB and SILCYE expression\(^{44}\). During papaya fruit ripening, CpNAC2 co-operated with CpEIN3a to promote CpPDS2/4, CpZDS, CpCHYB, and CpLCYE transcription, accounting for the elevated carotenoid contents\(^{17}\). CcNAC1/2 were transcriptionally upregulated under red-light treatment in Citrullus colocynthis\(^{45}\). Similarly, FcrNAC22 upregulated carotenoid metabolism and ABA synthesis via activation of FcrLCYB1, FcrBCH2 and FcrNCED5 in RL-irradiated fruits\(^{46}\). Here, we observed that the increased expression levels of NAC56 (the SINAC48 homolog) and NAC100 mediated by RL were positively related with expression of up-stream genes (PSY, Z-ISO2, ZDS6, LCYB and LCYE) in carotenoid metabolic pathway, while downregulated NAC68/78/90 showed a negative correlation.
with transcript for these genes, consistent with lycopene accumulation in ripening grapefruit fruit (Fig. 2, 5 and 7; Fig. S4).

Some publications in recent years have reported that MYB TFs played a positive role in carotenoid regulation. In the flavedo of *Citrus reticulate*, CrMYB68 indirectly inhibited the transformation of α/β-carotene via negative regulation for *CrBCH2* and *CrNCED5*\(^{15}\). AdMYB7 was positively correlated with *AdLCYB* in terms of expression and further regulated carotenoid biosynthesis\(^{19}\). In ‘darkred’ module, we found that MYB308 induced by RL was also positively correlation with carotenoid accumulation, especially lycopene, during grapefruit ripening (Fig. 4 and 5). Additionally, seven WRKY TFs were differentially expressed in response to RL during grapefruit ripening (Fig. 5). In *Osmanthus fragrans*, OfWRKY3 was found to be a positive regulator of the *OfCCD4* gene via binding to its W-box palindrome motif\(^{47}\). In this study, we also observed that two WRKY TFs, namely WRKY7/55 were gradually down-regulated as grapefruit fruit ripening, accompanied by the reduction of *CCD4-2* expression of in RL treatment (Fig. 2, 5 and 7). Besides, RL also notably suppressed expression for C3H (2), bZIP (4) and Dof (2) TFs in ‘brown’ module, suggested these TFs might involve in carotenoid accumulation (Fig. 5).

**Conclusion**

In present study, the carotenoid accumulation in grapefruit respond differently to light quality, RL have the significant inducing role during fruit ripening. The process was modulated by multiple TFs (bHLH74/91/122, NAC56/78/90/100, MYB/MYB308,
WRKY7/55, MADS29/AGL61, ERF043/118,) as well as carotenogenic genes (PSY, Z-ISO2, ZDS6, LCYB, LCYE, CHYB, CCD1-1/1-3, CCD4-2 and NCED2/3), and a preliminary regulatory model of red light-induced carotenoid metabolism in grapefruits was established (Fig. 7). These findings not only provide new insight into the regulation of carotenoid metabolism, but also offer an effective approach for enhancing the quality of citrus fruits in agricultural practice.

Materials and methods

Plant materials and treatments

‘Huoyan’ grapefruit were cultivated at the National Citrus Germplasm Repository of the Citrus Research Institute at the Chinese Academy of Agricultural Sciences in Chongqing, China and used as experimental materials. Trees with the same age, tree structure, and identical growth conditions were selected for the experiment and cultivated under the same management condition. Grapefruits with similar sizes and colors from outside of tree were bagged with four different light-transmitting paper bags at 120 days after blossom (DAB)—red-light-transmitting bags (RL) (peak wavelength, 748 nm), blue-light-transmitting bags (BL) (peak wavelength, 478 nm), and white-light-transmitting bags (WL), and a dark-shading bag (DS) was as the control (Fig. S1). Fruits of a uniform size were picked at 185 (maturation) and 215 (fully ripe) days after blossom (DAB). Each fifteen fruits were as one replicate and three biological replicates were used for each sample point of every treatment. After determining the basic physiological parameters, the fruits were cut into small cubes, frozen using liquid
nitrogen, and stored at −80 °C for further analysis.

**Determination of basic physiological parameters**

The fruit color parameters were measured using the Hunter Associates Laboratory Scanner (Hunter Associates Laboratory, Inc., Reston, VA, USA). The citrus color index (CCI) for the mesocarp was calculated according to the formula $CCI = 1000 \times \frac{a^*}{(L^* \times b^*)}$, using five fruits as a single replicate and three biological replicates were used for each sample. To determine the total soluble solid (TSS) content, 200 μL of fresh squeezed juice was obtained from juice sacs and then analyzed with a digital hand-held refractometer (Atago PR-101R, Atago, Japan). Titratable acidity (TA) was measured after the juice sample was diluted 50 times with purified water.

**Extraction and identification of carotenoids**

Carotenoids were identified following our previously described method. Ten grams of pulp powder was extracted with 20 mL of solvent (hexane/acetone/ethanol, 50:25:25, v/v/v) in a screw-top tube. The colored top layer was recovered and dried with nitrogen gas after being left to stand for 30 min, protected from light. After saponification, 2 mL of 1% butylhydroxytoluene (BHT)/methyl tert-butyl ether (MTBE) was added to the colored layer, and the mixture was filtered through sodium sulfate into a brown bottle for drying. The residue was dissolved in 2 mL of methanol/acetone (2:1, v/v) for HPLC analysis.

The carotenoids were identified by HPLC (Waters, Milford, MA, USA) with a C30
chromatography column (250 × 4.6 mm, 5 μm; YMC, Wilmington, NC, USA). The mobile phases for the carotenoids were composed of MTBE (A), methanol (B), and an aqueous phase (C) and were prepared by a multistep linear gradient elution. The identification was performed by comparing the retention times and UV–visible spectral peaks between the samples and standards. The carotenoid contents were calculated according to a standard curve based on authentic compounds and are expressed herein as mg/kg fresh weight (FW).

Library construction, transcriptome sequencing, and gene annotation

Total RNA was extracted using an Agilent RNA 6000 Nano kit (Agilent, CA, USA) according to the manufacturer’s instructions, the RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyzer, and the OD260/OD280 and OD260/OD230 values were determined using a NanoDrop 2000 spectrophotometer (NanoDrop 2000, Wilmington, DC, USA) to assess the RNA purity. Eight mRNA libraries were constructed for RNA-seq of the pulp samples harvested at 185 and 215 DAB. Three biological replicates were performed for each sample.

The libraries were sequenced on an Illumina HiSeq™ 2000 system at the Beijing Genomics Institute (BGI), China. The raw sequencing data were filtered by removing adaptors, low-quality and redundant sequences, and reads with unknown “N” base content higher than 5% using the SOAPnuke (version 1.4.0) and Trimmomatic (version 0.36) software. The clean reads were aligned to the reference genome database using HISAT (version 2.1.0)\textsuperscript{49}. 

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For transcription factor annotation, open reading frames (ORF) were obtained from the quality-checked data using getorf (EMBOSS: 6.5.7.0, http://emboss.sourceforge.net/apps/emboss/apps/getorf.html, -minsize 150) and aligned to Plant Transcription Factor Database (http://planttfdb.gao-lab.org/blast.php).

Identification of differentially expressed genes (DEGs)

The RSEM software package (version 1.2.8, http://deweylab.biostat.wisc.edu/rsem/rsem-calculate-expression.html) was used to calculated expression levels for transcripts with the default parameters. The expression levels are expressed as FPKM values. The genes that were differentially expressed between two samples were determined as previously described according to the Poisson distribution and the algorithm developed by BGI. The DEGs (p ≤ 0.005; |log2 fold change| ≥1; FDR ≤ 0.001) were then screened for further analysis.

Weighted gene co-expression network analysis (WGCNA) and network visualization for candidate genes

A total of 16831 unigenes with FPKM values > 1 were utilized to conduct weighted gene co-expression network analysis using WGCNA, reshape2 and stringr packages in Rstudio (v1.4.1717, https://www.rstudio.com/products/rstudio/download/). To reduce the size of the data calculation, a total of 12619 unigenes with the first 75% variance were screened from the above unigenes with unsigned TOM type to build a co-expression network. The phenotypic data regarding the carotenoids in the pulp were
associated with the constructed co-expression network to screen the modules that were significantly correlated with carotenoid metabolism ($p \leq 0.05$). Finally, DEGs were imported into the cytoscape software (version 3.7.2, https://cytoscape.org/download.html) for network visualization.

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**Data availability**

The transcriptome raw reads have been deposited as a BioProject (https://submit.ncbi.nlm.nih.gov/subs/sra/) under accession number: PRJNA728380.

**Author contributions**

W.X. designed the project. X.H. prepared the manuscript. L.H. analyzed the data. W.K. and W.Z participated in collecting the materials. C.Y., L.H., and X.H. participated in assaying the physiological parameters.

**Competing interests**

The authors declare no competing interests

**Additional information**

Supplementary information
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**Figures and figure legends**
Fig. 1 Effect of grapefruit bagged with four light-transmitting bags on the TSS content (A), TA content (B) and CCI (C) of grapefruits during ripening. Error bar indicate standard error from three biological replicates (n = 3). DS: dark-shade treatment; RL: red-light treatment; BL: blue-light treatment; WL: white-light treatment. Different letters indicate statistically significant difference in one-way ANOVA analysis.
Fig. 2 Effects of different light treatments on content of carotenoid, including β-carotene, phytofluene, ζ-carotene, lycopene, and 9-cis-violaxanthin content during grapefruit ripening. Error bars indicate the standard error from three biological replicates (n = 3). DS: dark-shade treatment; RL: red-light treatment; BL: blue-light treatment; WL: white-light treatment. Different letters indicate statistically significant difference in one-way ANOVA analysis.
Fig. 3 Profiling the changed genes and DEGs between grapefruit at different ripening stages. (A) The histogram presents the number of upregulated and downregulated genes between samples during grapefruit ripening. (B) Venn diagram for DEGs between grapefruit samples at two ripening stages. “A” is the control group and “B” was the treatment group in “A-vs-B”. DS: dark-shade treatment; RL: red-light treatment; BL: blue-light treatment; WL: white-light treatment.
Fig. 4 Weighted gene co-expression network analysis of grapefruit during ripening under different light-transmittance treatments. A. Hierarchical clustering tree displays twenty-four modules of co-expressed genes, in which each leaf represents one gene. B. Modules related to carotenoid and corresponding $p$-values. The left panel indicates twenty-four modules and the number of genes contained by each module. The right panel displays a color scale for module or trait correlations from $-1$ to $1$. 
Fig. 5 Heatmap analysis of structural genes (A) and transcriptional factors (B) correlated
with carotenoid metabolism during grapefruit ripening. Rows and columns indicate gene names and samples in the heatmap, respectively. Red, white and green represent high, medium and low expression level for genes. DS: dark-shade treatment; RL: red-light treatment; BL: blue-light treatment; WL: white-light treatment.

**Fig. 6** The co-expression network of TFs and structural genes related to carotenoid metabolism. Dot sizes and colors represent the numbers for correlated genes.
Fig. 7 The proposed models of carotenoid metabolism mediated by red light during grapefruit ripening. Pale pink and light green rounded rectangle, respectively, represents up- and down-regulated TFs in response to RL. Red and green arrow indicate positive and negative regulation of TFs on structural genes, respectively. Oval suggests...
structural genes in carotenoid metabolic pathway. Different colors represent up- and down-regulation level for genes in response to RL.
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