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Research paper

Transcriptomic and physiological comparison of Shatangju (*Citrus reticulata*) and its late-maturing mutant provides insights into auxin regulation of citrus fruit maturation

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Previous studies have shown that abscisic acid (ABA) and ethylene are involved in pulp maturation and peel coloration in the nonclimacteric citrus fruits. There are also signs indicating that other plant hormones may play some roles in citrus fruit ripening. In this study, we compared profiles of genome-wide gene expression and changes in hormones and peel pigments between fruits of Shatangju mandarin (*Citrus reticulata* Blanco, designated WT) and its natural mutant, Yuenongwanju (designated MT). The MT fruit matures ~2 months later than the WT fruit. Significant differences in fruit diameter, total soluble solids, titratable acid content, chlorophylls and carotenoids were detected between the fruits of the two genotypes at the sampled time points. Genome-wide transcriptome profiling showed that many genes involved in auxin and ABA metabolism and/or signaling pathways were differentially expressed between the MT and the WT fruits. Importantly, the expression of *CrYUCCA8* was significantly lower and the expression of *CrNCED5* was significantly higher in WT than in MT fruits at 230 and 250 DPA, respectively. In addition, the indole-3-acetic acid (IAA) level in the MT fruit was significantly higher than that in the WT counterpart, whereas a significantly lower level of ABA was detected in the mutant. Treatment of the WT fruit with exogenous IAA significantly delayed fruit maturation. Our results provide experimental evidence supporting the notion that auxin is a negative regulator of fruit maturation in citrus.

Keywords: Citrus fruit, auxin, carotenoid, maturation, transcriptome.

Introduction

The maturation of citrus fruit is coupled with significant changes both in peel color and in pulp sugar and acid content. The process is believed to be triggered and regulated by external and internal stimuli (Iglesias et al. 2001). Known plant hormones involved are ethylene, abscisic acid (ABA) and gibberellins (GAs) (Stewart and Wheaton 1972, Porat et al. 2001, Rodrigo et al. 2003). Ethylene does not trigger an autocatalytic ethylene production and a corresponding respiration peak in the non-climacteric citrus fruits as it does in apple, banana and other climacteric fruits. Yet, ethylene does trigger color changes in citrus fruit peel (Stewart and Wheaton 1972, Sawamura 1981, Baldwin and Biggs 1983, Katz et al. 2004, Porat 2008). Exogenous ethylene treatment accelerated chlorophyll degradation in citrus fruits (Stewart and Wheaton 1972). Reduced ethylene production was found in some late-ripening mutants along with lower expression of ethylene biosynthesis genes, such as *ACC synthase* and *ACC oxidase* (Wu et al. 2014, Terol et al. 2019). Ethylene involvement in citrus fruit maturation is also manifested by its regulatory effects on the expression of carotenoid biosynthetic genes, chlorophyllase genes and other fruit ripening–related genes (Jacob-Wilk et al. 1999, Zhu et al. 2021). Increase

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in the ABA content was found to be concomitant with color development and carotenoid accumulation in peels of maturing citrus fruit (Rodrigo et al. 2003, Zhu et al. 2020). Upregulation of ABA biosynthesis and signaling genes were observed during citrus fruit maturation (Romero et al. 2012, Wu et al. 2014). Exogenous ABA treatment accelerated fruit coloration in Ponkan, whereas treatment with nordihydroguaiaretic acid, a synthetic ABA inhibitor, retarded fruit coloration and juice acid degradation (Wang et al. 2016). An ABA-deficient sweet orange [*Citrus sinensis* (L.) Osbeck] mutant, Pinalate, displayed slowed fruit degreening (Rodrigo et al. 2003). The content of ABA and pigments was lower in the flavedo of a stay-green mutant of Ougan (*Citrus reticulata* Blanco) in comparison with that in the motherwood Ougan (Zhu et al. 2020). Comparative transcriptional and proteomic analyses between late-ripening sweet orange mutants and their corresponding wild types also showed an essential role of ABA in citrus fruit maturation (Wu et al. 2014, Zhang et al. 2014). In contrast to the promotive effects of ethylene and ABA, GA treatment delays the color break (CB) of citrus fruits. Applications of GA to the fruit of an early maturity pomelo-grapefruit hybrid could effectively retain the fruit color and prevent postharvest fruit senescence (Porat et al. 2001). Pre- or postharvest treatment of fruits with gibberellic acid retarded pigment changes in Satsuma mandarin (*Citrus reticulata* Blanco) (Garcia-Luis et al. 1986).

Auxins have been suggested to be important for the attainment of ability to ripen and for the coordination in the subsequent steps of the ripening process of both climacteric (Yuan and Carbaugh 2007, Tatsuki et al. 2013, Pan et al. 2015) and nonclimacteric fruits (Böttcher et al. 2010, Chen et al. 2016). The content of indole-3-acetic acid (IAA), the primary natural auxin, was particularly high during fruit set and started to decline before the onset of fruit ripening (Symons et al. 2012, Teribia et al. 2016). Application of exogenous auxins delayed the fruit ripening process in strawberry (Chen et al. 2016), tomato (Cohen 1996), banana (Vendrell 1969) and grape (Davies et al. 1997). However, an initial increase in IAA content is necessary for stimulating ethylene production and fruit maturation in climacteric fruits such as apple (Yuan and Carbaugh 2007), plum (Farcuh et al. 2019), peach (Trainotti et al. 2007, Tatsuki et al. 2013) and pear (Kondo et al. 2004). The IAA also regulates carotenoid biosynthesis by repressing the expression of *phytoene synthase* (*PSY*), *ζ-carotene isomerase*, *phytoene desaturase* (*PDS*) and *carotenoid isomerase*, and promoting the expression of *β-cyclase 1* and *β-carotene hydroxylase* during tomato fruit ripening (Su et al. 2015). Impairment in IAA biosynthesis and signaling could also result in abnormal fruit ripening (Wang et al. 2005, Reig et al. 2018). A recent report showed that the exogenous application of NAA accelerated chlorophyll degradation and carotenoid accumulation in Satsuma mandarin fruit treated with two color-retarding agents, GA and prohydrojasmon (PDJ) (Ma et al. 2021).

Extending fruit maturation will extend its marketable season and hence increase the value of the fruit. Shatangju is very popular among citrus consumers in China for its superior fruit quality. The main drawback of the cultivar is its short fruit marketing period. Previously, we identified a mutant of Shatangju mandarin (*C. reticulata* Blanco, WT), designated as 'Yuenongwanju' (MT). Fruit maturation of the MT was significantly delayed by ∼50–60 days compared with that of the WT. Here, by comparing the transcriptomes and the contents of multiple metabolites and two phytohormones (ABA and IAA) of the MT and WT fruits, we provide insight into the role of IAA in citrus fruit maturation.

Materials and methods

Plant materials

Six-year-old Shatangju (WT) and its late-maturing mutant Yuenongwanju (MT) trees grafted onto red tangerine (*Citrus tangerina* 'Red Tangerine') rootstock were grown in the same greenhouse equipped with an insect-proof net at the orchard of the Institute of Fruit Tree Research, GDAAS (113.379 E, 23.159 N, Tianhe District, Guangzhou, Guangdong Province, China).

The WT and MT fruits were collected at 180, 210, 230, 250 and 270 days post-anthesis (DPA) and 180, 210, 230, 250, 270 and 300 DPA, respectively, in 2017 and 2018. The WT fruit reached full-size green (MG), CB and color changing (CC) at ∼210, 230 and 250 DPA, whereas the MT fruits reached the corresponding stages at 250, 270 and 300 DPA, respectively. The MT fruits were still green at 230 DPA and were therefore classified as the premature green stage (PMG) fruit. All fruit samples were washed with tap water once and with sterile, distilled water twice. Peels and pulps were separated and were frozen immediately in liquid nitrogen and were then stored at −80 °C until use. Fruits from three trees were collected as a biological replicate, and three replicates were used.

Determination of total soluble solid and titratable acid contents in juice pulp

The total soluble solid (TSS) content of the juice pulps was determined by using a refractometer (Pocket refractometer PAL-1, ATAGO Co., Ltd, Japan) and was referred to as °Brix. The titratable acid (TA) content was determined by a modified procedure of Widodo et al. (1996). In short, 10 ml of juice from each fruit sample was titrated with a 0.1 M NaOH solution. The TA was expressed as grams of TA per 100 ml of citrus juice.

Determination of chlorophyll in fruit peel

The concentrations of Chl a, Chl b and total chlorophyll in fruit peels containing flavedo and albedo were measured according to Xie et al. (2014). Briefly, fruit peel samples (0.2 g) were ground in liquid nitrogen into a fine powder and were then extracted three times with 3 ml of 80% acetone

(v/v). Centrifugation was performed at 4500 r.c.f. under 4 °C for 5 min on an Eppendorf 5430R centrifuge. The extracts were brought to a final volume of 10 ml with 80% acetone. The absorbance of each supernatant was measured at 645 and 663 nm in a DR3900UV–visible spectrophotometer (Hach Co.). Three biological replicates were measured.

Determination of total carotenoids in fruit peels

The extraction and analysis of total carotenoids in fruit peels containing flavedo and albedo were performed following Rehman et al. (2018). Fruit peel samples (0.25 g) were weighed and ground to powder in liquid nitrogen. The powder was transferred to a 50-ml centrifuge tube with 25 ml of *n*-hexane-acetone-methanol (50:25:25, v/v). Centrifugation was performed at 4500 r.c.f. under 4 °C for 5 min using an Eppendorf 5430R centrifuge. The supernatant was transferred to a new 50-ml centrifuge tube, and 10 ml of NaCl (10%) was then added. The top-colored portion was taken to determine the absorbance at 450-nm wavelength using a DR3900UV–visible spectrophotometer (Hach Co.). The total carotenoid content was calculated using the linear regression equation of the standard curve that was established with the standard β-carotene (Sigma–Aldrich, St Louis, MO, USA, File S1 available as Supplementary data at *Tree Physiology* Online), and the absorbance of the supernatant was measured at 450 nm.

Peel transcriptome profiling

Only the WT fruit peels collected at 210, 230 and 250 DPA and the MT fruit peels collected at 230, 250, 270 and 300 DPA were subjected to RNA-seq analysis using an Illumina sequencing platform at the Genedenovo Biotechnology Co., Ltd (Guangzhou) following their standard procedures. The mRNA preparation included the following three steps: total RNA extraction, ribosomal RNA (rRNA) removal, poly(A) + RNA (mRNA) purification and mRNA quality assessment. First-strand cDNA was synthesized from the fragmented mRNA template by using a ProtoScript M-MuLV First Strand cDNA Synthesis Kit (NEB, MA, USA) with random primers. Double-stranded cDNA was synthesized from the first-strand cDNA template using DNA polymerase I. Purification, end repair/dA-Tailing and ligation of sequencing adapters to the cDNAs were completed by using corresponding reagent kits. Approximately 200-bp long cDNAs were isolated and purified by using AMPure XP beads (Beckman Coulter, High Wycombe, UK) following the manufacturer's instructions.

The RNA-seq was completed on an Illumina HiSeq2500. OpenGene/fastp was employed to filter and remove the low-quality reads from sequencing data (Chen et al. 2018). The clean reads were first aligned to the *Citrus clementina* genome and then to all transcript sequences. Bowtie2 was employed to remove the rRNA sequences from the clean reads using the Ribosomal database (Langmead and Salzberg 2012). A value of fragments per kilobase of transcript per million mapped reads (FPKM) was calculated for quantitative gene expression analysis. The differentially expressed genes (DEGs) between WT and MT fruits were identified by using DESeq2 (Love et al. 2014). Only the DEGs with FDR < 0.05 and $|\log_2\text{Fold Changes}| > 1$ were subjected to Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Principal component analysis (PCA) was performed with the R package to reveal the dimensions of transcriptomic data from different fruit development stages.

RNA extraction, first-strand cDNA synthesis and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from fruit peel samples using an RNAprep Pure Plant Plus Kit (Tiangen, Beijing) following the manufacturer's instructions. The quality and concentration of the extracted total RNA were analyzed by electrophoresis on a 1.0% (w/v) agarose gel and by using a NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA synthesis was performed using a PrimeScript RT Reagent Kit with gDNA Eraser (RR047A, Takara, Dalian).

The expression of a subset of representative DEGs was validated by applying RT–qPCR. The primers for the DEGs and the reference gene *Actin* were designed by employing Primer3 software (https://primer3.ut.ee/). The primer sequences are listed in Table S1 available as Supplementary data at *Tree Physiology* Online. A total of 20 μl of PCR mixture (SYBR green PCR mix, Applied Biosystems), containing both primer pairs of the target and reference genes, was used for each sample. Thermal cycling was performed on an ABI 7500 Real-Time System (Applied Biosystems) programmed at an initial incubation at 50 °C for 2 min and an immediate denaturing at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative gene expression level was normalized against the level of the *CrActin* gene and was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Determination of ABA and IAA contents in juice pulps

The extraction and analysis of ABA and IAA were performed at Wuhan MetWare Biotechnology Co., Ltd (www.metware.cn). Fifty milligrams of the frozen powder of citrus pulp were ground in liquid nitrogen, transferred to centrifugal tubes (Corning, MA, USA) and vortexed for 10 min at 4 °C after adding 0.5 ml of extraction solution. Centrifugation was performed at 14,000 r.p.m. at 4 °C for 5 min, and the supernatant was collected. The pellet was extracted again and combined and was then dried by evaporating at 35 °C under a nitrogen stream. The dried extracts were added to 80% methanol (V/V), dissolved by ultrasonic treatment, filtrated and diluted to a constant volume.

The separation and detection of hormones were performed on an LC–ESI–MS/MS system following standard procedures. The qualitative analysis of ABA and IAA in the mass spectrometry data was carried out for each sample based on the

MetWare database constructed with plant hormone standards. The ABA and IAA data were identified and corrected via their characteristic absorption spectra and the typical retention time by using Analyst 1.6.1 (AB Sciex).

In vitro treatment of MG Shatangju fruits with IAA

The MG Shatangju fruits were collected and washed with tap water to remove the dust. Surface sterilization was performed by first immersing the fruits in 70% ethanol for 3 min, followed by consecutively washing each, for 30 s, with 70% ethanol and sterilized water. Fruits were further sterilized in 0.1% carbendazim for 10 min after air-drying and were rinsed in sterile distilled water three times. The fruits were air-dried at room temperature.

The IAA was dissolved in a small amount of absolute ethanol and was diluted in water to either 1000 or 100 μM. The above surface-sterilized fruits were immersed in IAA solutions and were vacuum-infiltrated under −15 to −20 kPa for ∼2 min. The fruits were removed and placed in the dark at 25 ± 2 °C and at a relative humidity of 50–70% after the vacuum was slowly withdrawn.

Statistical analyses

Relative gene expression and juice pulp ABA and IAA contents were analyzed by employing one-way ANOVA with SigmaPlot software for Windows (version 11.0, Systat Software Inc., Erkrath, Germany). A significance level of $P < 0.05$ using Student's t test was employed for the analysis of genes' relative expression and hormone level. All data are presented as the mean ± standard error ($n = 3$).

Results

Temporal changes in TSS, TA, chlorophyll and total carotenoids in WT and MT fruits

The fruits from WT and MT trees at various stages of development are shown in Figure 1. As can be seen, the most pronounced difference between MT and WT fruits was the onset of peel color changes. The WT fruits started changing color at ∼250 DPA when the MT fruits were still green. The MT fruit only began to change color at 300 DPA and therefore lagged behind the WT fruit by 50–60 days. We also compared the longitudinal and the transverse diameters of the WT and MT fruits at various stages of growth and development. The results clearly showed that the MT fruits were exclusively smaller than the WT fruits at the same early and middle developmental stages until the MT fruits reached the stage of CC (Figure 1B).

We also monitored the changes in TSS and TA in juice sacs and the changes in chlorophylls and carotenoids in the peels of WT and MT fruits. The trends showed generally a steady increase in TSS content and a slow decrease in TA content in both the WT and the MT fruit pulps during maturation (Figure 1C). The TSS contents were significantly higher in WT fruit than in MT fruit at 210, 230, 250 and 270 DPA, whereas the TA contents were significantly higher in MT fruits than in WT fruits only at two time points, the 180 and the 210 DPA. As shown in Figure 1D, the contents of chlorophyll a, chlorophyll b and total chlorophyll steadily decreased and the contents of total carotenoids gradually increased in both WT and MT fruits as they matured. The contents of chlorophyll a, chlorophyll b and total chlorophyll were significantly higher in MT than in WT at 230, 250 and 270 DPA. By contrast, the total carotenoid contents were significantly higher in WT than in MT at 230, 250 and 270 DPA.

Transcriptome changes in WT and MT during fruit development and maturation

The peel tissues of both WT (210, 230 and 250 DPA) and MT (230, 250, 270 and 300 DPA) fruits were subjected to genome-wide gene expression analysis by using the RNA-seq technique. The PCA scatter plot of the transcriptome data is shown in Figure 2A. Clearly, the fruits could be separated by the physiological development stages by PC1. More specifically, fruits at the MG stage, i.e., the MT fruits of 250 DPA and the WT fruits of 210 DPA, were grouped together by PC1. Similarly grouped were the fruits at the CB stage (MT of 270 DPA and WT of 230 DPA) and the fruits at the CC stage (MT of 300 DPA and WT of 250 DPA). Not surprisingly, the PMG fruit (MT of 230 DPA) was singled out by PC1. The results indicated that fruits of different physiological development stages had quite different/characteristic gene expression patterns. The PC2 axis was also able to separate the MT and the WT fruits from two early development stages, MG (210 DPA of WT and 250 DPA of MT) and CB (230 DPA of WT and 270 DPA of MT), but not the fruits from the later stage of CC when both the MT and the WT fruits had already changed peel color.

The DEG profiles also showed a pattern similar to that obtained by PCA analysis. As shown in Figure 2, the numbers of the DEGs were smaller when the WT and the MT fruits from the same physiological development stages (Figure 2B, WT 210 DPA vs MT 250 DPA; Figure 2C, WT 230 DPA vs MT 270 DPA; Figure 2D, WT 250 DPA vs MT 300 DPA) were compared. By contrast, the DEG numbers were more than doubled when both the WT and the MT fruits at the same sampling dates were compared (WT 230 DPA vs MT 230 DPA; WT 250 DPA vs MT 250 DPA). For example, only 567 upregulated and 333 downregulated genes were found in fruits in the CC stage (Figure 2D, WT 250 DPA vs MT 300 DPA), whereas 1868 upregulated and 1214 downregulated genes were identified when the fruits of WT and MT collected at the same 230 DPA were compared (Figure 2E). Similarly, 2238 upregulated and 810 downregulated genes were identified when the fruits

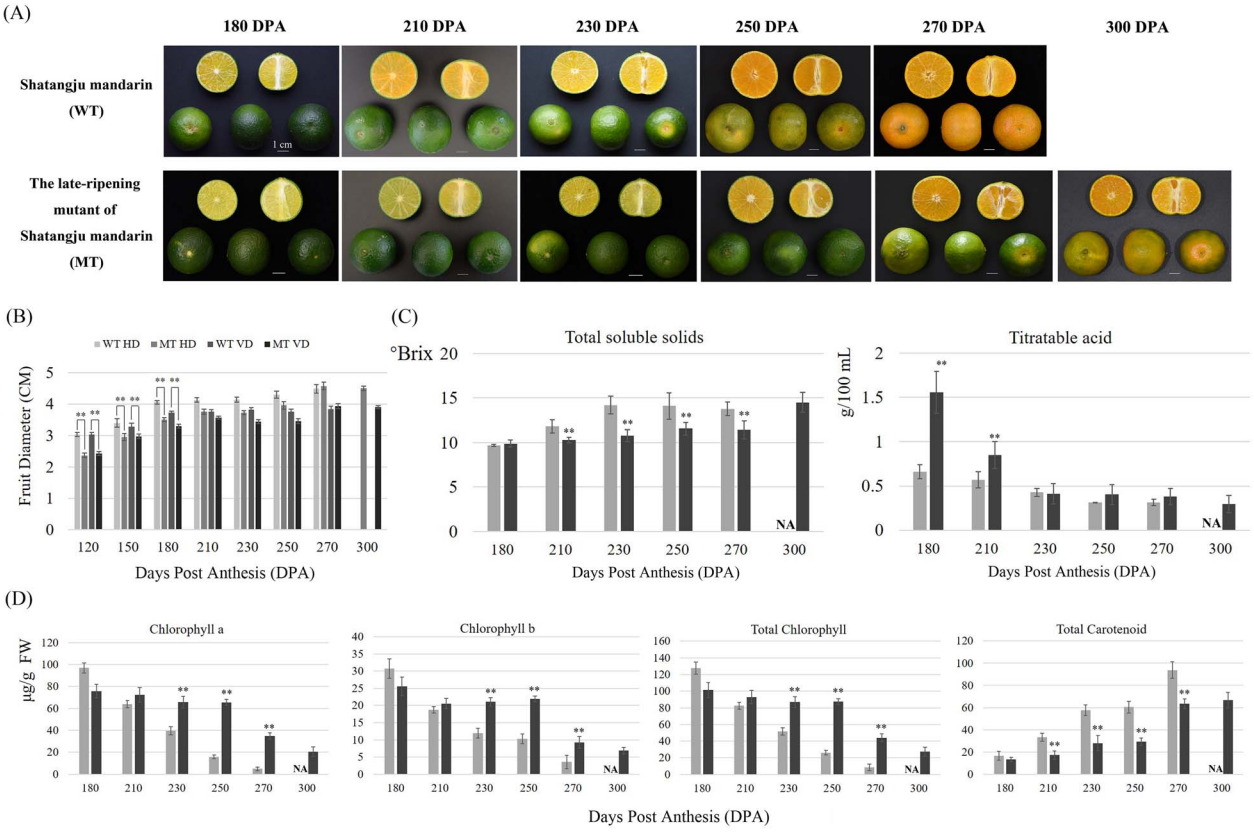

Figure 1. Characters of Shatangju (WT) and its spontaneous mutant Yuenongwanju (MT) fruit during development and maturation. (A) Phenotypic characterization of fruits. (B) The horizontal diameter (HD) and vertical diameter (VD) of fruits. The flowers were tagged with date of flower opening. The diameters of fruits were measured by using a Vernier caliper at different days post-anthesis (DPA). Values represent means ± SE (n = 10). (C) TSS and TA contents in juice pulp. (D) Chlorophyll and total carotenoids contents in fruit peels. Values represent means ± SE (n = 3) in C and D. Asterisks (**) indicate significant difference between two groups ($P < 0.05$) in all figures. NA in C and D means no WT fruits at 300 DPA were sampled for the analysis.

of both genotypes collected at 250 DPA were compared (Figure 2F). The underlying logic was that the development of WT and MT fruits was not physiologically synchronized, and MT fruits always developmentally lagged behind WT fruits.

The distributions of DEGs across relevant biological pathways were further classified by KEGG pathway analysis (Figure 2G) and GO enrichment analysis (Figure S1 available as Supplementary data at *Tree Physiology* Online). The results showed that most DEGs were metabolism-related, as they were assigned to 'carbohydrate metabolism', 'biosynthesis of secondary metabolites', 'amino acid metabolism', 'lipid metabolism' and 'energy metabolism'. Many DEGs were genetic information processing–related, as they were assigned to 'folding, sorting and degradation', and were environmental information processing–related, as they were assigned to 'signal transduction', or were organismal systems–related, as they were assigned to 'environmental adaptation'. The expression of 10 DEGs was quantified to verify the RNA-seq results by using RT–qPCR. As shown in Figure S2 available as Supplementary data at *Tree Physiology* Online, the overall trends in the expression of the analyzed genes were generally consistent between the RNA-seq and RT–qPCR data, showing that the quality of our RNA-seq data was acceptable.

Differences in the expression of auxin metabolic and signaling genes between WT and MT fruits

A total of 32 DEGs involved in IAA metabolism and signaling pathways were identified from our RNA-seq data. The DEGs with higher expression abundance was further analyzed (Figure 3). Among them were three *CrYUC* genes, *CrYUCCA3*, *CrYUCCA5* and *CrYUCCA8*, that encode proteins catalyzing the rate-limiting step in the IAA biosynthesis pathway (Figure 3A). Particularly, the *CrYUCCA8* was the most highly expressed of the three. The abundance of the *CrYUCCA8* transcripts was 10- to 20-fold higher than that of the *CrYUCCA5*. Temporally, the expression of *CrYUCCA8* steadily decreased as the fruit size increased in both genotypes. However, the expression level of the gene was always lower in WT than in MT at 230 and 250 DPA. The *CrDAO1*, a citrus homolog of *Arabidopsis AtDAO1* that is involved in IAA oxidation and homeostasis (Porco et al. 2016), was expressed significantly higher in MT than in WT at 230 DPA and 250 DPA (Figure 3B). Although six *GH3* genes,

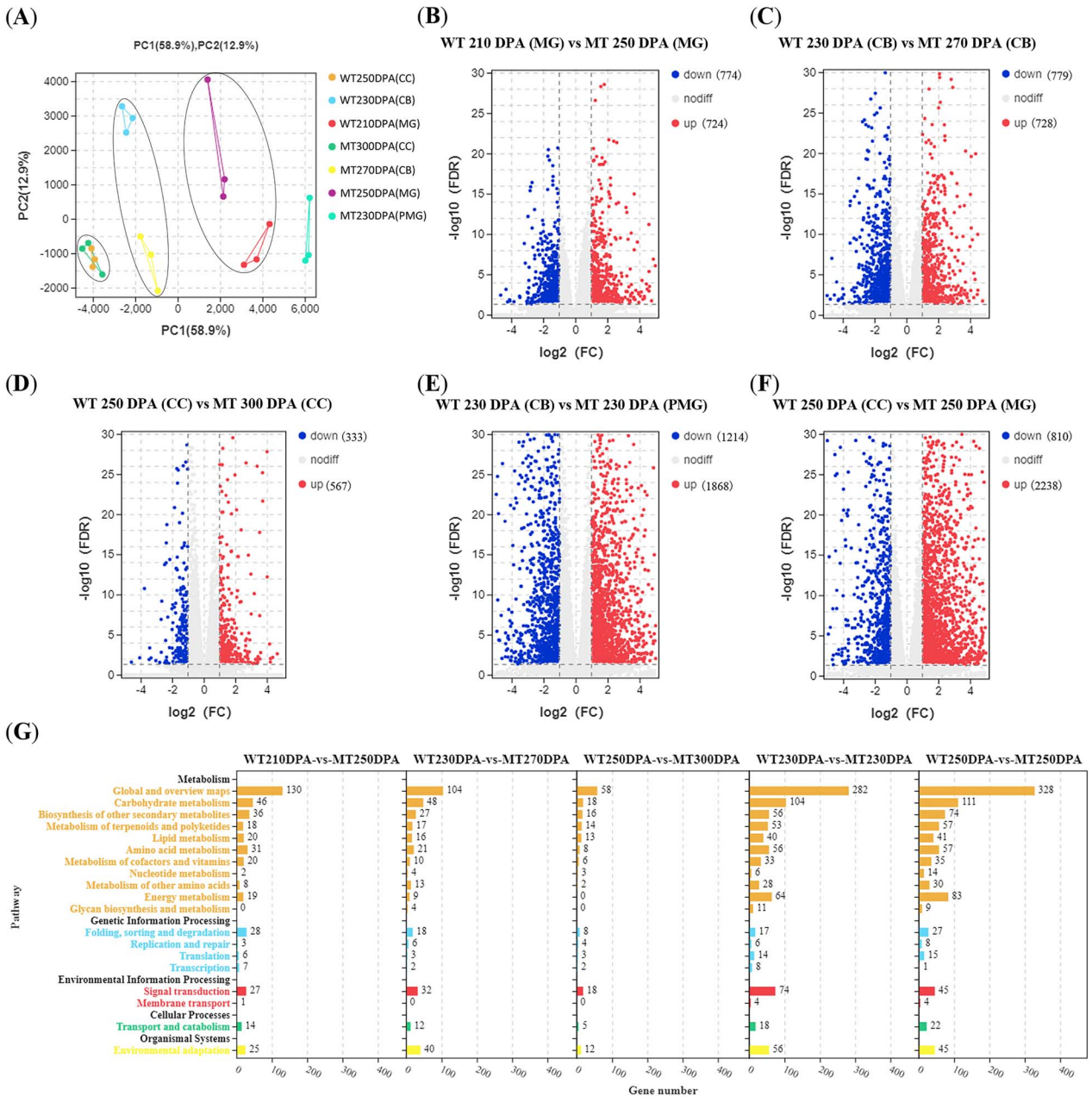

Figure 2. Transcriptome differences in the peels of WT and MT fruits. (A) The PCA of transcriptome data at different development and maturation stages. (B–F) Volcano map of DEGs between WT and MT fruits collected at different development and maturation stages. (G) KEGG analysis of DEGs. Three biological repetitions were used to conduct RNA-seq analysis. The numbers in figure were the numbers of DEGs assigned to different metabolic pathways between WT and MT.

responsible for conjugating free IAA to amino acids to reduce the free IAA level in plants, were shown to be DEGs by RNA-seq data (Figure 3C and Figure S3A available as Supplementary Data at *Tree Physiology* Online), only one of them, *CrGH3.01*, was abundantly expressed in the fruits of both genotypes. The remaining five *GH3* genes were always <1 FPKM in abundance, indicating they might play a very limited role in the IAA conjugation process. The *CrGH3.01* seemed to have experienced a moderate decrease as fruit growth proceeded. Nevertheless, a much higher expression level was observed in MT fruits (5.43 and 6.29 FPKM) than in WT fruits (3.31 and 4.10 FPKM) at 230 and 250 DPA, respectively.

Since the auxin levels were significantly different between the young WT and MT fruits, we investigated the expression of genes involved in the auxin signaling pathway. The results showed that the expression of two auxin influx carrier genes, *CrLAX1* and *CrLAX2*, which belong to the *AUX1/LAX* family, were significantly higher in MT fruits than in WT fruits at

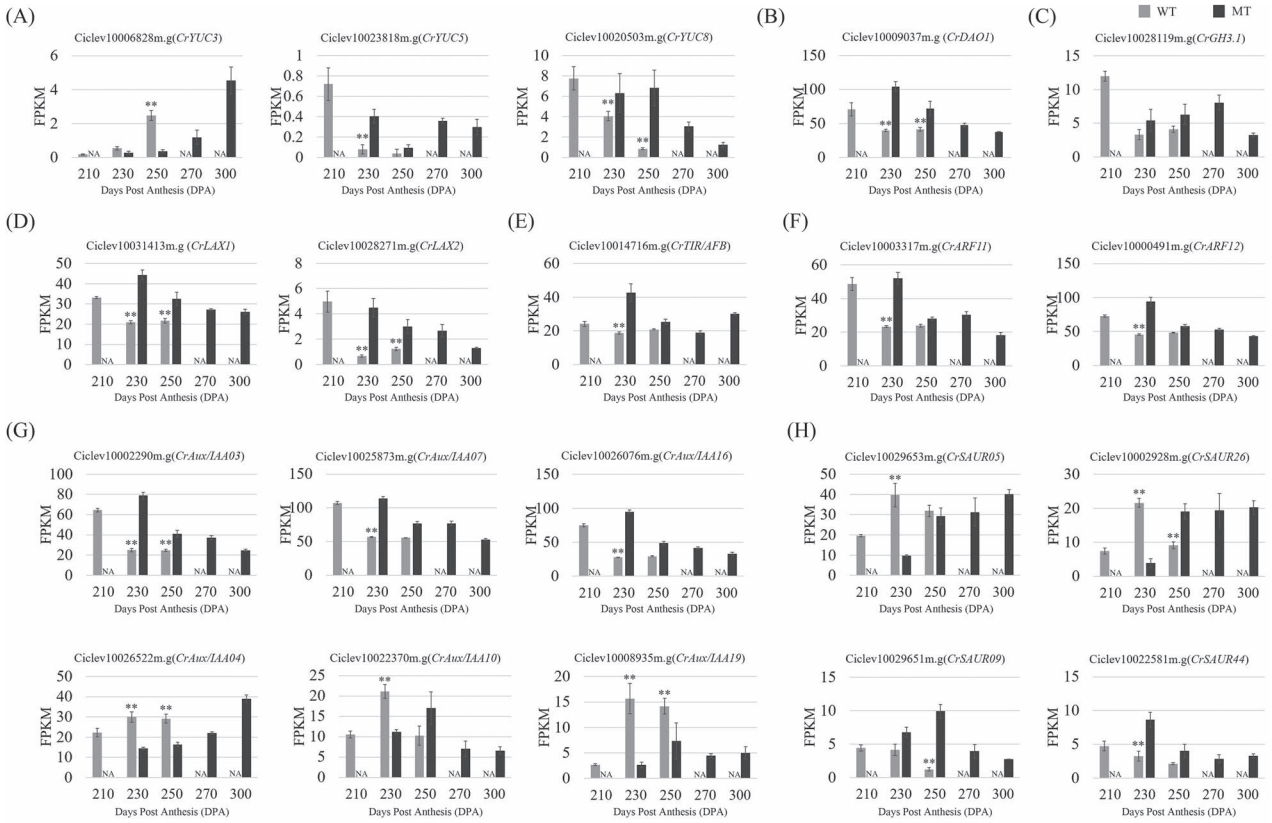

Figure 3. Differentially expressed genes in auxin metabolism and signaling pathway of both genotypes. (A) The expression pattern of *CrYUCs* genes. (B) The expression pattern of *CrDAO1* gene. (C) The expression pattern of DEGs of *GH3* gene. (D) The expression pattern of *CrLAX1* and *CrLAX2* gene. (E) The expression pattern of *CrTIR* gene. (F) The expression pattern of auxin response factor family gene *CrARFs*. (G) The expression pattern of DEGs of *AUX/IAA* gene. (H) The expression pattern of DEGs of *SAUR* gene. Value represent means ± SE ($n = 3$). Asterisks (**) indicate significant difference between the two groups ($P < 0.05$). NA means no WT or MT fruits were subjected to RNA-seq analysis.

230 and 250 DPA (Figure 3D). The *CrLAX1* should be more important in the IAA transport than *CrLAX2* since *CrLAX1* transcript abundance was higher than that of *CrLAX2* at all investigated time points. An auxin receptor gene, *TIR/AFB* (Ciclev10014716m.g), was expressed significantly lower in WT fruits than in MT fruits at 230 DPA (Figure 3E), suggesting that the response to auxin was weaker in WT fruits than in MT fruits at the investigated time point. Four auxin response factor (*ARF*) gene family members, *CrARF11*, *CrARF12*, *CrARF20* and *CrARF21*, were differentially expressed between WT and MT. The *CrARF11* and *CrARF12* were expressed significantly lower in WT than in MT at 230 DPA, resembling the expression of the auxin receptor gene *TIR/AFB* (Figure 3F and Figure S3B available as Supplementary data at *Tree Physiology* Online).

Twelve *CrAux/IAA* family genes encoding auxin early response proteins, which interact with *ARFs* exhibited differential expression, and six highly abundantly expressed members were analyzed (Figure 3G). The *CrIAA03*, *CrIAA07* and *CrIAA16* were expressed at lower levels in WT fruits than in MT fruits at 230 and 250 DPA. By contrast, *CrIAA04* and *CrIAA19* were expressed at higher levels in WT fruits than in MT fruits at 230 and 250 DPA. The *CrIAA10* expression was higher at 230 DPA and lower at 250 DPA in WT fruits than in MT fruits.

The *SAUR* family has a central role in auxin-induced acid growth. Eight *SAUR* members were identified as DEGs between WT and MT fruits (Figure 3H and Figure S3C available as Supplementary data at *Tree Physiology* Online). The expression of *CrSAUR05* and *CrSAUR26* was low in small fruits, i.e., in MT fruits at 230 DPA and in WT fruits at 210 DPA, but was rapidly increased and maintained at high level at later stages of fruit development. However, the expression of *CrSAUR26* decreased in WT fruit at 250 DPA when the fruits were changing color. It should be mentioned that *CrSAUR05* and *CrSAUR26* were the most abundantly expressed members of the *SAUR* family. The other two less abundantly expressed *SAUR* family DEGs, *CrSAUR09* and *CrSAUR44*, showed a steady decrease in their expression in both WT and MT fruits as the fruits grew.

Changes in expression of ABA biosynthesis and signaling genes in WT and MT fruits

The ABA is a fruit ripening regulator in *Citrus*. The ABA level is largely dependent on the activity of 9-*cis*-epoxycarotenoid dioxygenase (NCED), the rate-limiting enzyme in ABA

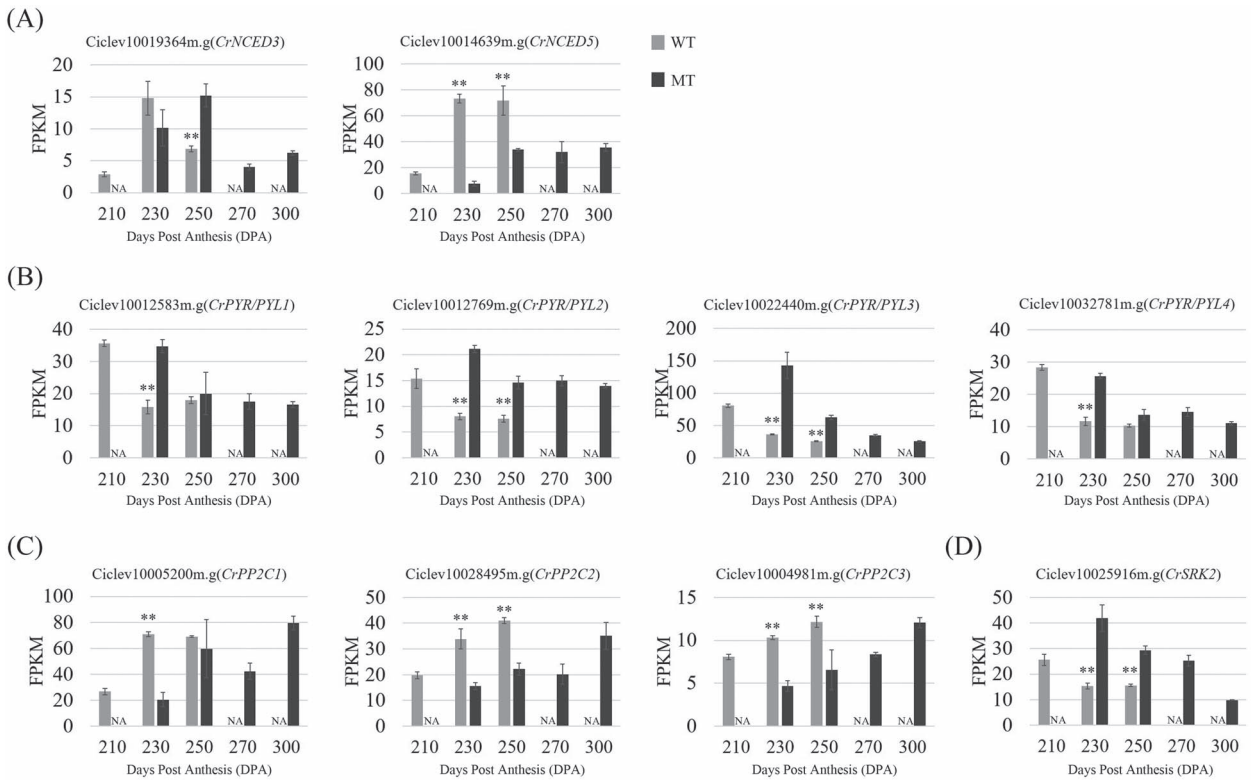

Figure 4. Differentially expressed genes in ABA biosynthesis and signaling pathway of both genotypes. (A) The gene expression pattern of *CrNCEDs* genes. (B–D) The expression pattern of DEGs involved in ABA signaling pathway. Values represent means ± SE (n = 3). Asterisks (**) indicate significant difference between the two groups ($P < 0.05$). NA means no WT or MT fruits were subjected to RNA-seq analysis.

biosynthesis in higher plants. We found that the transcription of two *CrNCED* family genes, *CrNCED3* and *CrNCED5*, was significantly different between WT and MT fruits (Figure 4A). The *CrNCED5* expression experienced a sharp increase in young fruits, and afterward, it was maintained at a relatively high level in both the WT and the MT fruits. Specifically, its expression level increased from 15.55 to 73.19 FPKM in WT fruits between 210 and 230 DPA and from 7.69 to 33.87 FPKM in MT fruits between 230 and 250 DPA. Another NCED gene, *CrNCED3*, followed approximately an inverted V curve in its expression in fruits of both genotypes, although its transcript abundance was much higher in WT fruits than in MT fruits at 230 DPA.

Differences were observed in the expression of eight ABA signaling pathway genes between WT and MT fruits. The expression of *CrPYR/PYL1*, *CrPYR/PYL2*, *CrPYR/PYL3* and *CrPYR/PYL4*, which are ABA receptor genes (*PYR1/PYL/RCAR*), was significantly lower in WT fruits than in MT fruits at 230 DPA. The expression differences were reduced for all four genes but were still significant for *CrPYR/PYL2* and *CrPYR/PYL3* at 250 DPA (Figure 4B). The immediate downstream genes *CrPP2C1*, *CrPP2C2* and *CrPP2C3*, which encode Type 2C protein phosphatases, all showed a significantly higher transcription level in WT fruits than in MT fruits at 230 DPA. The expression difference disappeared for *CrPP2C1* but still existed for *CrPP2C2* and *CrPP2C3* at 250 DPA (Figure 4C). One member from the *SnRK2* family, *CrSRK2* (Ciclev10025916m.g), was expressed at a significantly lower level in the WT fruits than in MT fruits at 230 and 250 DPA (Figure 4D).

Differentially expressed genes in the metabolism of carotenoids and chlorophylls

Seven DEGs in carotenoid metabolism and eight DEGs in chlorophyll metabolism were identified between WT and MT. The *CrPSY2* gene, which encodes phytoene synthase, a major rate-limiting enzyme in carotenoid biosynthesis, had significantly higher expression in WT fruits (67.05 FPKM) than in MT fruits (31.58 FPKM) at 230 DPA. The *CrPSY2* expression was reduced at 250 DPA in WT fruits that were changing color (38.78 FPKM). By contrast, the expression of the gene continuously increased in MT fruits at 250 (48.44 FPKM) and 270 DPA (69.42 FPKM) but decreased at 300 DPA (54.93 FPKM) at which the fruits were changing color. The ζ-carotene desaturase gene continuously increased its expression as the fruit grew in both genotypes. However, its expression level was significantly higher in WT (87.80 and 106.61 FPKM) than in MT (47.65 and 53.27 FPKM) at 230 and 250 DPA. The expression of *CrLCYb*, a lycopene β-cyclase gene, continuously increased as the fruits developed and peaked in maturing fruits, i.e., in WT fruits (140.52 FPKM) of 250 DPA and in MT fruits (151.60 FPKM) of 300 DPA. Notably, the gene had

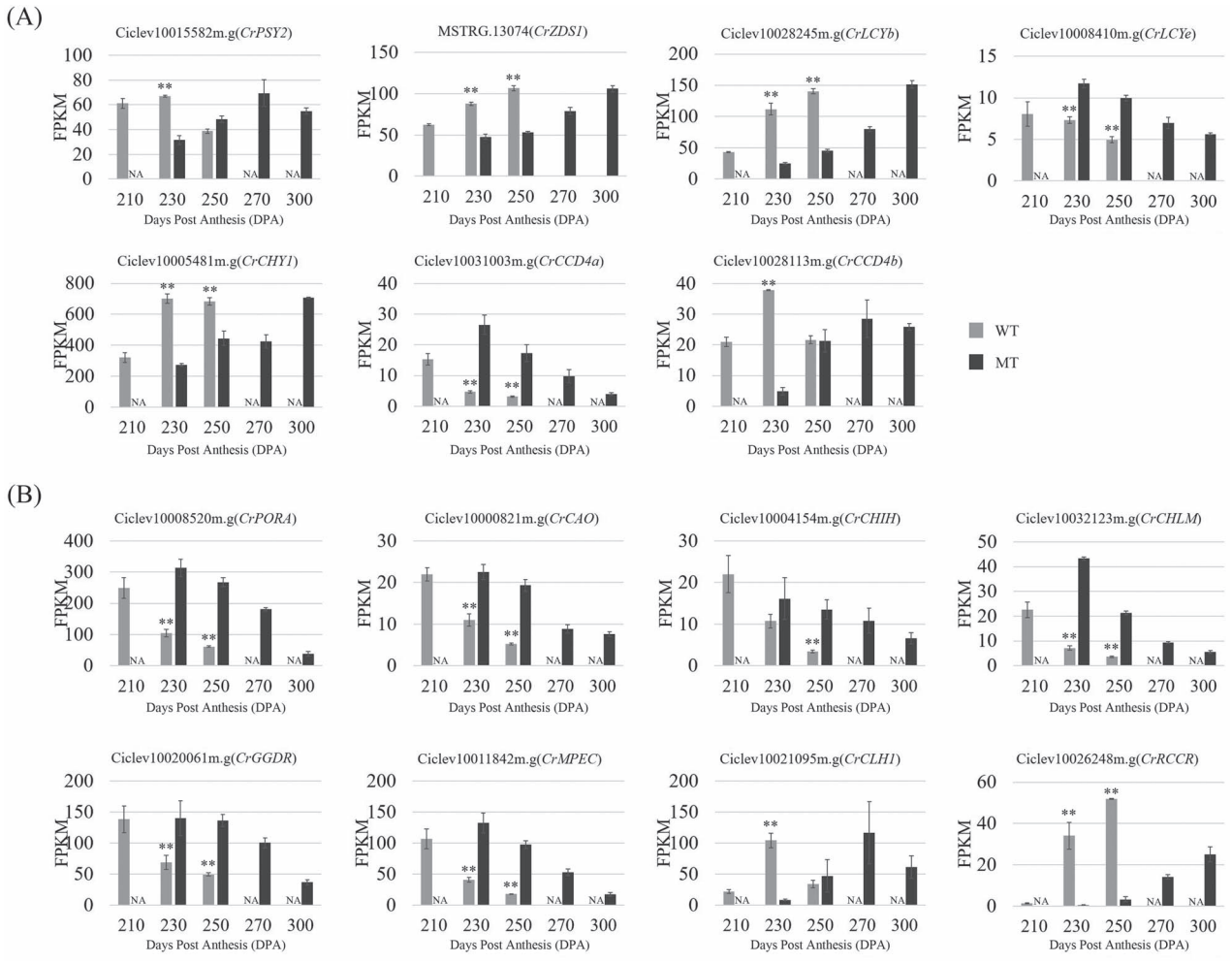

Figure 5. Differentially expressed genes in carotenoid and chlorophyll metabolism pathway of both genotypes. (A) The expression pattern of DEGs that assigned to carotenoid metabolism pathway. (B) The expression pattern of DEGs that assigned to chlorophyll metabolism pathway. Values represent means ± SE ($n = 3$). Asterisks (**) indicate significant difference between the two groups ($P < 0.05$). NA means no WT or MT fruits were subjected to RNA-seq analysis.

a significantly higher expression in WT than in MT at 230 and 250 DPA. Unlike *CrLCYb*, *CrLCYe* continuously decreased in its expression in developing fruits and reached the lowest expression level in maturing WT (4.97 FPKM) and MT fruits (5.64 FPKM) at 250 and 300 DPA, respectively. Comparatively, its expression was significantly lower in WT fruits than in MT fruits at both 230 and the 250 DPA. The expression of a *CHYB* gene (*CrCHY1*) steadily increased in both the MT and the WT fruits as fruits developed and reached peak expression in WT (683.83 FPKM) and MT (706.12 FPKM) at 250 and 300 DPA, respectively. Its expression was significantly higher in WT fruits than in MT fruits at 230 and 250 DPA. Two members of the carotenoid cleavage dioxygenase 4 (CCD4) gene family that act negatively in the pathway, *CrCCD4a* and *CrCCD4b*, were differentially expressed between WT and MT fruits. The expression of *CrCCD4a* gradually decreased during fruit development in both genotypes and reached the lowest level at 250 DPA in WT (3.17 FPKM) and at 300 DPA in MT (4.00 FPKM), respectively. Its expression was significantly lower in WT than in MT at both 230 and the 250 DPA. The expression of *CrCCD4b* increased in green fruits and then slightly decreased in the maturing fruits of both genotypes. Yet, a very significant difference in the expression of the gene was shown between the WT and the MT fruits at 230 DPA, for the gene's transcripts were ~7.7 times more in WT (37.82 FPKM) than in MT (4.89 FPKM) (Figure 5A).

We detected six chlorophyll biosynthesis genes and two chlorophyll degradation genes as the DEGs between WT and MT fruits (Figure 5B). The six genes, *CrPORA*, *CrCAO*, *CrCHIH*, *CrCHLM*, *CrGGDR* and *CrMPEC*, showed a steady decreasing trend in their expression as fruits developed and reached the lowest expression point in CC fruits of both genotypes (Figure S4 available as Supplementary data at *Tree Physiology* Online). Nevertheless, they were all expressed at significantly higher levels in WT than in MT at 230 and 250 DPA. The two chlorophyll degradation genes, *CrCLH1* and *CrRCCR*, increased

in their expression as the fruits developed. The *CrCLH1* expression experienced a very significant increase in WT fruits from 210 (21.93 FPKM) to 230 DPA (104.57 FPKM) and in MT fruits from 230 (8.55 FPKM) to 270 DPA (116.83 FPKM), and then a moderate decrease in WT fruits at 250 DPA and in MT fruits at 300 DPA, which were changing color. The *CrRCCR* showed a continuous upregulation in its expression in the growing fruits of both genotypes, but its transcript abundance was significantly higher in WT than in MT at both 230 and the 250 DPA.

Exogenous IAA treatment delayed color change in WT fruits

Consistent with the expression trend of the DEGs involved in the metabolism of ABA and IAA, a gradual increase in the ABA level and a steady decrease in the IAA level were observed in immature fruits of both genotypes. As shown in Figure 6A, an ABA peak was observed at the color change stage in both the MT and the WT fruits. There was no significant difference between the ABA peaks of the two genotypes. However, the ABA contents were unexceptionally lower in all MT fruits at 180, 210, 230 and 250 DPA than in the WT fruits of the same ages. Figure 6A also showed the changes in fruit endogenous free IAA content in both genotypes. It was clear that the fruit free IAA content gradually decreased in both genotypes as the fruit size increased. The IAA content stabilized at a lower level, ∼0.6 ng g^{-1} FW, after the fruits reached full size. Comparatively, the MT fruits contained significantly higher free IAA than did the WT fruits at all time points prior to 250 DPA.

The higher free IAA content in the MT fruit prompted us to investigate whether IAA was responsible for the delayed maturation of the MT fruits. The MG WT fruits, ∼210 days postanthesis, were therefore picked and treated in vitro with IAA in 2019 and 2020 (Figure 6B). As expected, the content of free IAA in fruits treated with IAA was significantly higher than that in water-treated and untreated fruits (CK). Treatments with 100 and 1000 μM of IAA increased the pulp IAA levels by ∼15- and 25-fold, respectively, within 24 h. The IAA concentrations in fruits of both treatments decreased rapidly but were still significantly higher than those in the controls after 48 h (Figure 6C). It was not unexpected that the IAA-treated WT fruits exhibited a significant delay of color change (Figure 6D). Both the untreated CK and the ddH_2O-treated fruits completely turned yellow after 25 days of storage at RT. Meanwhile, the fruits under 100 μM IAA treatment had just started changing color, and those under the 1000 μM IAA treatment were still deep green (Figure 6D, upper panel). At 60 days posttreatment, fruits treated with 100 μM IAA had almost completely changed color, but those treated with 1000 μM IAA had only slightly changed color (Figure 6D, lower panel).

Discussion

Fruit ripening is controlled by different hormones. For example, the ripening of climacteric fruits such as tomato, peach, banana and papaya is regulated by ethylene and IAA (Vendrell 1969, Trainotti et al. 2007, Su et al. 2015, Cai et al. 2022). Ripening of nonclimacteric fruits, such as strawberry, grape and sweet cherry, is predominantly regulated by ABA and auxin (Jia et al. 2011, Wang et al. 2015, Pilati et al. 2017). Auxin, unlike its positive role in enhancing the biosynthesis of ethylene in climacteric fruits (Trainotti et al. 2007, Su et al. 2015), works as a negative regulator, inhibiting ABA biosynthesis in nonclimacteric fruits (Gu et al. 2019). Citrus fruits are classified as nonclimacteric fruits. Their ripening process is therefore considered to be mainly controlled by ABA, although accumulating evidence has suggested that ethylene plays some role, at least in chlorophyll degradation in fruit peel (Trebitsh et al. 1993).

The role of IAA in citrus fruit ripening has not been extensively explored. In this study, a late ripening mutant of Shatangju mandarin whose fruits mature in February, almost 2 months later than common Shatangju, was characterized. It was found that the younger fruit contained more free IAA and less ABA than did the older fruit, suggesting that IAA might act as a counter maturation agent (Figure 6A). Furthermore, exogenous IAA treatment significantly delayed color change in WT fruits (Figure 6D). Additionally, significant differences in either IAA or ABA levels existed between the MT and the WT fruits of the same age. That is, the IAA content was higher, whereas the ABA content was lower, in the MT fruit than in the WT fruit. These differences between the two genotypes no longer existed only in fruits that were experiencing color change. Taken together, it seemed that a higher level of free IAA and a lower level of ABA were necessary for maintaining the growth of the citrus fruit until color change. Intriguingly, a match in the IAA or ABA content could always be found between an older MT fruit and a younger WT fruit. In other words, the MT fruits were able to reach the same physiological status of the WT fruits later (20–30 days), indicating that a slower growth in the early stages of the MT fruit development should be responsible for the late-ripening phenotype. Contrary to our findings, a synthetic auxin, NAA, was found to be able to accelerate chlorophyll degradation and carotenoid accumulation in GA- and PDJ-treated Satsuma mandarin fruit (Ma et al. 2021). Nevertheless, studies have shown that NAA had bipolar effects on citrus, as manifested by the fact that it either prevents fruitlet drop if used at lower concentrations or promotes fruitlet drop if used at higher concentrations (Stover et al. 2002).

The fruit of the late-maturing mutant exhibited a significant delay in CB and a dramatic change in the expression of numerous genes. The most pronounced internal changes were that the IAA level was elevated, whereas the ABA level was lowered in growing fruit by the mutation. Correspondingly, some of the IAA and ABA metabolic genes were differentially regulated. Endogenous free IAA levels were determined collectively by the genes of IAA biosynthesis, conjugation and degradation pathways (Korver et al. 2018). The indole-3-pyruvate (IPA) pathway was considered to be the

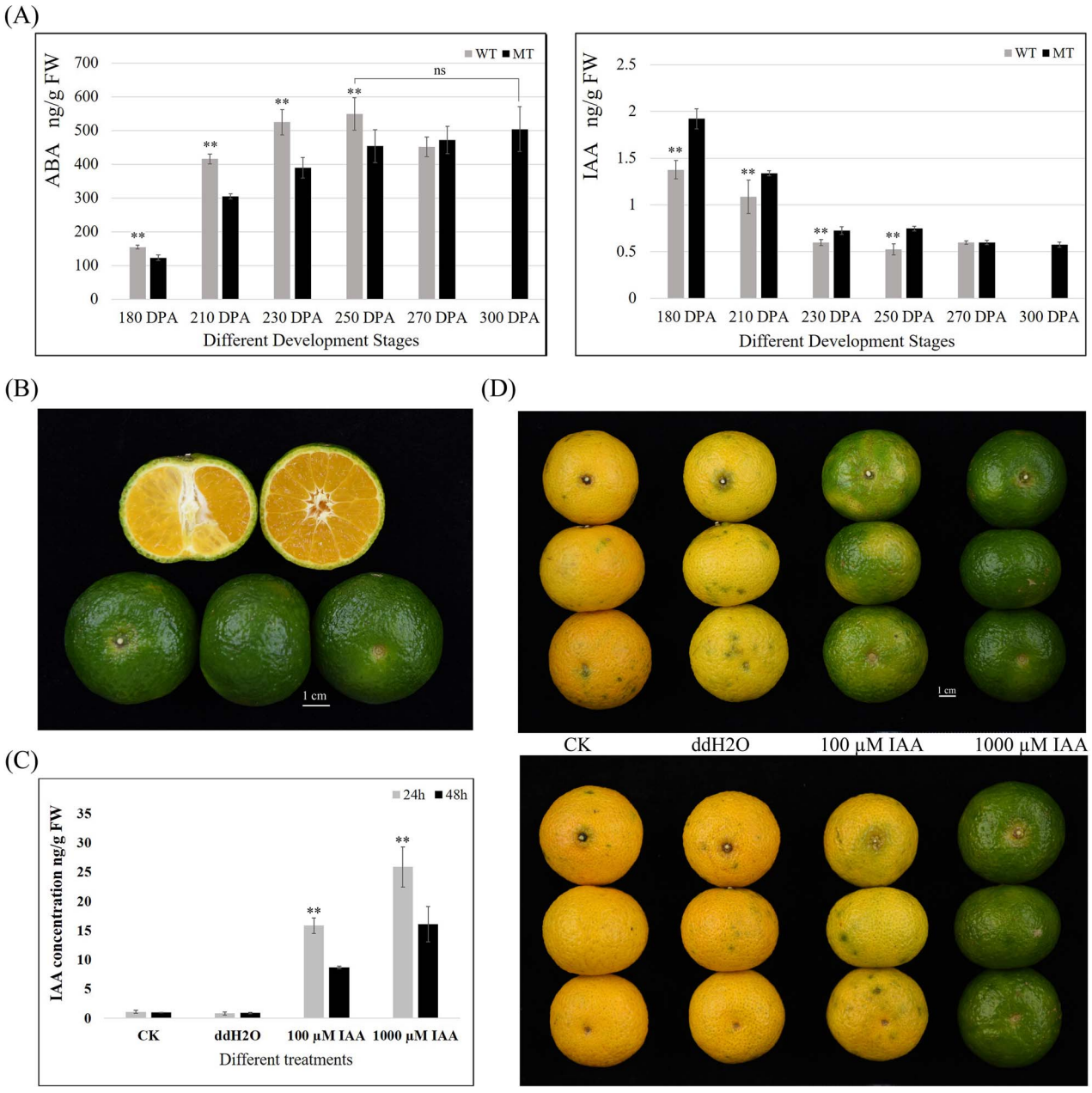

Figure 6. Effect of exogenous IAA on the color change of detached Shatangju green fruits. (A) Free IAA and ABA contents in the fruit pulp of Shatangju and its spontaneous mutant Yuenongwanju detected using a LC-ESI-MS/MS system. NA means no WT fruits at 300 DPA were sampled for the analysis. (B) The MG citrus fruits collected at 210 DPA stage. (C) The content of IAA in fruits at 24 h and 48 h after treatment. Fruits without treatment were used as control (CK). (D) Phenotypes of CK fruits and the fruits treated with ddH_2O, 100 and 1000 μM IAA after 25 days (up) and 60 days (down) of storage. Values represent means ± SE (n = 3). Asterisks (**) indicate significant difference between two groups ($P < 0.05$), whereas 'ns' denotes no significant difference.

predominant pathway for IAA biosynthesis in higher plants (Mashiguchi et al. 2011). The rate-limiting step of the pathway that catalyzes the conversion of IPA to IAA is controlled by the *YUCCA* gene family (Zhao et al. 2001), and three members of the family, *CrYUCCA3*, *CrYUCCA5* and *CrYUCCA8,* were found to be expressed differentially between WT and MT (Figure 3A). Although the expression of *CrYUCCA3* was opposite to that of *CrYUCCA8*, the three genes combinedly showed a decrease in their expression, which was in agreement with the trend in changes in free auxin. An auxin degradation gene, *DAO1*, was abundantly, but differentially, expressed between WT and MT, and remarkably, its expression was twice as high in MT as in WT at 230 DPA (Figure 3B). It seemed that the expression of a *GH3* gene was also elevated in MT fruits (Figure 3C). Logically, the elevation in the expression of IAA degradation and conjugation genes should lead to a reduction in the IAA levels in MT fruits, but our findings were the opposite. Multiple studies have shown that alterations in the auxin levels could be

corrected by the compensatory changes in auxin metabolism since auxin homeostasis is vital for maintaining normal cell function (Barbez et al. 2012). For example, *Arabidopsis* plants lacking *AtDAO1* activity did not result in an increase in free IAA levels since the conjugation of IAA was concomitantly elevated (Mellor et al. 2016, Porco et al. 2016). For ABA biosynthesis, the rate-limiting step that cleaves xanthoxin to produce 9-*cis*-violaxanthin and 9-*cis*-neoxanthin is controlled by the *NCED* gene family, and two members of the family, *CrNCED3* and *CrNCED5*, were found to be expressed differentially between WT and MT (Figure 4A). Remarkably, the expression level of *CrNCED5* (Ciclev10014639m.g) in the MT fruit was only half that of the WT fruit at the last two physiological stages, the CB stage (230 DPA WT and 270 DPA MT fruit) and the CC stage (250 DPA WT and 300 DPA MT fruit). The *NCED5* gene was considered to be a key regulator in ABA biosynthesis in citrus fruit (Agustí et al. 2007, Zhu et al. 2020). The gene's expression was closely corelated to the content of ABA in the two studied genotypes (Figures 4A and 6A).

Fruit ripening is a complex biological and physiological process involving changes in many primary and secondary metabolic processes (Wu et al. 2014, Ohmiya et al. 2019, Zacarias and Rodrigo 2020). One of the pronounced changes is the fruit color. Citrus fruit's color development is correlated with a gradual reduction in green color and a steady increase in mainly orange and yellow colors (Porat 2008). Metabolic changes in chlorophylls and carotenoids therefore play a very important role in the coloration of citrus fruit peel (Ohmiya et al. 2019). Our RNA-seq data showed that the genes related to chlorophyll and carotenoid metabolisms were differentially expressed. Three genes, *CrPSY2*, *CrZDS1* and *CrLCYb2a*, which encode three key enzymes in the carotenoid biosynthesis pathway, expressed at higher levels in WT fruit than in MT fruit at 230 and 250 DPA (Figure 5A). Consistently, six genes, *CrPORA*, *CrCAO*, *CrCHIH*, *CrCHLM*, *CrGGDR* and *CrMPEC*, involved in the process of chlorophyll biosynthesis were more highly expressed, while two genes, *CrCLH1* and *CrRCCR*, involved in chlorophyll degradation were less expressed in WT than in MT (Figure 5B). Similarly, auxin could also affect the gene expression of carotenoid and chlorophyll metabolism during tomato fruit ripening (Su et al. 2015).

In this study, we did not measure the IAA content in fruit peels for technical reasons. Although peel coloration and pulp maturation are two different processes in the same ripening fruit, they are normally synchronized in the majority of the world citrus growing areas. Similarly, in our case, the changes in peel color were closely coupled with the changes in pulp sugar and acid content. It is therefore relatively safe to assume that the changes in IAA content in pulps should similarly occur in peels, and no fruit peel IAA data should interfere with drawing a reliable conclusion. It must be pointed out that the citrus fruit may delay or stop CC during fruit ripening in rare cases, such as in some very early satsuma mandarins, and in hot, tropical regions.

Conclusions

Our experimental evidence supports that IAA is a negative regulator in the fruit maturation process of citrus by delaying carotenoid accumulation and chlorophyll degradation. Fruit free IAA content is negatively correlated with fruit age. By contrast, fruit ABA content is positively corelated with fruit age. The late-ripening mutant of Shatangju mandarin (MT) exhibits a lower fruit growth rate and an extended fruit growth period as compared with its mother cultivar, Shatangju mandarin (WT). The fruits of the MT plants are therefore physiologically younger than their peers of the WT plants. Thus, the MT fruit contains higher levels of IAA and lower levels of ABA than the WT fruit of the same age. The genes involved in IAA and ABA metabolisms and fruit color change are correspondingly regulated.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

All authors declare that there are no competing interests.

Data availability statement

Sequence files and metadata for all samples in the study have been deposited in the NCBI Sequence Read Archive repository, accession number PRJNA954525. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contribution

H.Y. and G.Z. conceived and designed the experiments. H.Y. and B.Z. collected the materials. H.Y., Y.L., S.R. and Y.Z. performed the experiments. B.W. and B.J. analyzed the data. H.Y. and G.Z. wrote the manuscript. C.J. guided on manuscript writing and additional experiments.

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