Original article

Comparative transcriptome analysis explores candidate genes related to carotenoid accumulation in pericarp of two pummelo cultivars (*Citrus grandis* L. Osbeck)

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Summary

Carotenoid has an important impact on the nutritional quality and colour development of fruits. Although the knowledge on carotenoid metabolism is well known, the mechanism of regulation of carotenoid accumulation remains unclear. 'Huangbao' pummelo (red-pericarp) is selected from bud mutation of 'Guanxi' pummelo (yellow-pericarp). In order to reveal the differences in colour development of fruits between the two cultivars, carotenoid contents and transcriptomic profile were analyzed in the pericarp of ripe fruits from the two pummelo cultivars. Results show that the lycopene content in the pericarp of 'Huangbao' ($160.29 \mu\text{g g}^{-1}$ FW) was significantly higher compared with 'Guanxi' ($0.91 \mu\text{g g}^{-1}$ FW). Lycopene is the main pigment responsible for pericarp colouration. A total of 929 differentially expressed genes (DEGs) were identified. Based on Gene Ontology (GO) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, some genes enriched in signaling pathway, carotenoid isomerase activity, and carbon fixation in photosynthetic organism pathway may be associated with carotenoid accumulation in the pericarp. Genes encoding for fructose-bisphosphate aldolase, enolase, and pyruvate kinase isozyme were significantly up-regulated in 'Huangbao', while genes annotated as phytoene synthase and prolycopene isomerase were distinctly down-regulated. Gibberellin-2- β -dioxygenase (GA2OX) gene was especially expressed in 'Huangbao'; however, genes encoding for DELLA protein and auxin transporter were not detected. *ERF* transcription factors involved in ethylene signaling pathway were down-regulated in 'Huangbao'. These findings are meaningful to reveal that carotenoid precursor metabolism and phytohormone pathways may be involved in the regulation of lycopene accumulation in the pericarp of pummelo fruits.

Keywords

carotenoid, *Citrus grandis* L. Osbeck, gene expression, red-pericarp, regulation, RNA-seq

Abbreviations

IPP, Isopentenyl pyrophosphate; GA, Gibberellin; DMAPP, Dimethylallyl pyrophosphate; MVA: Mevalonic acid pathway; MEP, Methylerythritol phosphate; GGP, geranylgeranyl diphosphate; PSY, Phytoene synthase; PDS, Phytoene desaturase; ZDS, ζ -carotene desaturase; CRTISO, Carotenoid isom-

Significance of this study

What is already known on this subject?

- Carotenoid composition in citrus fruits is closely associated with genotype. In an orange-pericarp mutant of 'Guanxi' pummelo, the distinct β -carotene accumulation in pericarp is accompanied by the suppression of downstream genes for β -carotene degradation. Several transcription factors identified in this mutant may be involved in the regulation of carotenoid accumulation.

What are the new findings?

- In this study, lycopene accumulation was obvious in the pericarp of fruits from 'Huangbao' pummelo (a red-pericarp mutant selected from 'Guanxi' pummelo). In the pericarp of 'Huangbao' fruits, carotenoid precursor metabolism was promoted, while two genes related to lycopene synthesis were unexpected down-regulated. Several genes involved in gibberellin metabolism, auxin transport, and ethylene signaling pathway may be involved in the regulation of lycopene accumulation.

What is the expected impact on horticulture?

- The results in this study provide meaningful information for revealing the regulatory mechanism involved in carotenoid accumulation in citrus fruits.

erase; β -LCY, Lycopene β -cyclase; ϵ -LCY, Lycopene ϵ -cyclase; BCH, β -ring carotene hydroxylase; ECH, ϵ -ring carotene hydroxylase; DEG, Differentially expressed gene; BP, Biological process; MF, Molecular function; CC, cellular component; FPKM, Fragments per kilobase of transcript per million mapped reads; GA2OX, Gibberellin-2- β -dioxygenase; ACS, 1-aminocyclopropane-1-carboxylate synthase; ACO, 1-aminocyclopropane-1-carboxylate oxidase; EBF, EIN3-binding F-box protein; EIN3, Ethylene insensitive 3; ABC, ATP binding cassette; qRT-PCR, quantitative real time reverse transcription PCR; ACC, 1-aminocyclopropane-1-carboxylate; IAA, Indole-3-acetic acid; CCD4, carotenoid cleavage dioxygenase 4; MTBE, Methyl tert-butyl ether; BHT, Butylated hydroxytoluene; HPLC, High performance liquid chromatography.

Introduction

Carotenoids are synthesized in plants and micro-organisms, playing important roles in growth and development of organisms. Carotenoids impact colour development in plant tissues, furthermore, act as one of the light harvesting pig-

ments, participating in the photosynthetic process in plants (Phillip and Young, 1995). The intermediate product in the carotenoid metabolic pathway is the precursor for abscisic acid and gibberellin (GA) biosyntheses (Song et al., 2016; Wang et al., 2017). Carotenoids can also improve oxidation resistance in plants due to their role in scavenging active oxygen species (Alboresi et al., 2011). More importantly, carotenoids are beneficial for human health. It is well known that carotenoids contribute to eye health (Abdel-Aal et al., 2013), reduce the risk of cancer (Fiedor and Burda, 2014), and stimulate osteoblastic bone formation (Yamaguchi, 2012). Therefore, carotenoid metabolism has gained much attention in recent years.

The knowledge on carotenoid metabolism is well known. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are synthesized through mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathways, which then act as precursors for carotenoid biosynthesis. The MVA and MEP pathways exist in the cytosol and plastids of plants, respectively. Carotenoids are synthesized mainly through MEP pathway in plants (Sponsel, 2002). IPP and DMAPP are catalyzed by geranylgeranyl pyrophosphate synthase to form geranylgeranyl diphosphate (GGPP). Phytoene synthase (PSY) catalyzes condensation of two molecules of GGPP, forming the phytoene. Phytoene is desaturated by phytoene desaturase (PDS) to yield ζ -carotene, and then the desaturation of ζ -carotene is catalyzed by ζ -carotene desaturase (ZDS) to produce tetra-cis-lycopene. Carotenoid isomerase (CRTISO) converts tetra-cis-lycopene to all-trans-lycopene. Lycopene β -cyclase (β -LCY) catalyzes the cyclization of all-trans-lycopene to form β -carotene. However, when the cyclization of all-trans-lycopene is catalyzed by β -LCY and lycopene ϵ -cyclase (ϵ -LCY), α -carotene is formed. β -ring carotene hydroxylase (BCH) and ϵ -ring carotene hydroxylase (ECH) catalyze the hydroxylation of α -carotene, yielding the lutein. β -carotene is hydroxylated only by BCH, converting to zeaxanthin. Zeaxanthin epoxidase converts zeaxanthin to antheraxanthin and violaxanthin. Violaxanthin is converted to neoxanthin by neoxanthin synthase at the last step (Nisar et al., 2015).

However, the regulation of carotenoid accumulation is not limited to the carotenoid biosynthetic pathway. Chromoplasts act as the sink for carotenoids, and undergo structural changes simultaneously with carotenoid accumulation (Schweiggert et al., 2011). The component analysis of chromoplasts in tomato (*Solanum lycopersicum*) fruits showed that the increased plastid-localized lipid monogalactodiacylglycerol content in chromoplast membrane was correlated with the promoted capacity for β -carotene and lycopene in chromoplasts (Nogueira et al., 2013). Some other metabolic pathways have been identified that are capable of impacting carbon skeleton supply for carotenoid biosynthesis or controlling carotenoid degradation, and consequently, influencing carotenoid accumulation (Wang et al., 2017). Therefore, a complex mechanism may be involved in the regulation of carotenoid accumulation in plants.

Carotenoid components are tightly associated with plant genotype. β -cryptoxanthin content in the pericarp of mandarin (*Citrus reticulata* Blanco) fruits was significantly higher compared to that of sweet orange (*Citrus sinensis* L. Osbeck) (10-fold), lemon (*Citrus limon* L.) (15-fold) and pummelo (*Citrus grandis* L. Osbeck) (200-fold) (Tao et al., 2003). Distinct differences in carotenoid contents could be found in different sweet orange cultivars, for instance, ζ -carotene and lutein mainly accumulated in 'Maltaise demi-sanguine'

fruits; however, lycopene was the main contributor to carotenoid accumulation in 'Meski Ahmer' fruits (Ben et al., 2018). A similar trend was also observed in vegetables (Smita et al., 2013) and flowers (Yoshioka et al., 2012). Therefore, it is necessary to carry out experiments using plant materials with different colour development patterns to explore further the mechanisms involved in regulation of carotenoid accumulation.

'Huangbao' pummelo was derived from the bud mutation of 'Guanxi' pummelo. The pericarp of the mutant-type fruits is characterized by the development of red colour during fruit ripening, while the wild-type pericarp remains yellow even at the fully ripe stage. The aim of the present study is to explore candidate genes related to carotenoid accumulation based on the investigation of transcriptome in the pericarp of ripe fruits from the two pummelo cultivars.

Materials and methods

Fruit samples

'Huangbao' was selected from bud mutation of 'Guanxi' pummelo (*Citrus grandis* L. Osbeck) for its red colour pericarp (Figure 1). 'Huangbao' and 'Guanxi' pummelo cultivars were both grafted on 'Sour pummelo' (*Citrus grandis*) and planted at 3.0 m $\times$ 3.5 m in a commercial orchard located in Zhangzhou City, China. Ripe fruit samples of each cultivar were harvested at the outside canopy of three 4-year-old trees with similar growth vigor on November 3, 2015. Three fruits were sampled from each tree and were considered as one biological replicate. The pericarp was cut into small pieces, mixed together and immediately frozen in liquid nitrogen, then stored at -80°C for RNA isolation and carotenoid estimation.

RNA preparation, cDNA library construction and RNA sequencing

Total RNA was extracted with E.Z.N.A. Plant RNA Kit (R6827-02) (Omega, Georgia, USA) following the manufacturer's instructions. A total amount of 3 μ g RNA per sample was used for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. The cDNA libraries were sequenced on an Illumina HiSeq 4000 platform.

Sequence quality control and unigene assembly

Raw data of fastq format were first processed through in-house Perl scripts. Low quality reads and reads containing adapter or poly-N were removed, and the remaining reads were selected as clean reads. The Q30 and GC-content of the clean reads were calculated. All of the analyses were based on clean reads with high quality sequences. The clean reads

FIGURE 1. Ripe fruits of 'Huangbao' (a) and 'Guanxi' (b) pummelo cultivars (*Citrus grandis* L. Osbeck).

were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive database (<https://www.ncbi.nlm.nih.gov/sra>) with accession number of SRP131839. The clean reads were used for *de novo* assembly using the Trinity software ([https://github.com/trinityrnaseq/wiki](https://github.com/trinityrnaseq/trinityrnaseq/wiki)) with min-kmer-cov set to 2 by default and all other parameters set default.

Functional annotation of unigenes

Alignment with an E-value threshold of $1e^{-5}$ against the following opening biological information databases: NCBI non-redundant protein sequences (NR) (<ftp://ftp.ncbi.nih.gov/blast/db/>), a manually annotated and reviewed protein sequence database (Swiss-Prot) (<http://www.uniprot.org/>), Clusters of Orthologous Groups (COG) (<http://www.ncbi.nlm.nih.gov/COG/>), euKaryotic Orthologous Groups (KOG) (<http://www.ncbi.nlm.nih.gov/KOG/>), Evolutionary Genealogy of Genes: non-supervised orthologous groups (eggNOG) (<http://eggNOGdb.embl.de/>), gene ontology (GO) (<http://www.geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>). Putative amino acid sequences of unigenes were BLAST searched against the Protein family database (Pfam) (<http://pfam.xfam.org/>) using HMMER software (<http://hmmer.org/>) with the e-value cutoff of $1e^{-10}$ to obtain the annotated information of unigenes.

GO and KEGG pathway enrichment analysis

GO enrichment analysis of DEGs was performed by the topGO software (R package v. 2.18.0) (<http://www.bioconductor.org/packages/release/bioc/html/topGO.html>) based Kolmogorov-Smirnov test. KO-Based Annotation System software (KOBAS2.0) (<http://kobas.cbi.pku.edu.cn/help.do>) was used to test the statistical enrichment of DEGs in KEGG pathways. Corrected P-value ≤ 0.05 was defined as significantly enriched in DEGs.

Differentially expression analysis of unigenes

Clean data were mapped back onto the assembled transcriptome, and the read count for each unigene was obtained from mapping. The transcript abundance of unigenes was statistically calculated using fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed unigenes were identified using the DESeq R package 1.10.1. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). A FDR threshold < 0.01 and fold change ≥ 2 were used to measure the significance of the unigene expression differences.

Carotenoid measurements

The procedure of carotenoid isolation was based on the method reported by Li et al. (2017) with some modifications. Briefly, carotenoids were extracted from 1.2 g of pericarp powder with 6 mL of n-hexane:acetone:ethanol (2:1:1, v/v/v) solution at 4°C for 20 h under dark conditions. The re-

maining pellet was suspended in 6 mL of extraction solution and maintained at 4°C for 4 h. The dried carotenoid extracts were dissolved in 1 mL of methyl tert-butyl ether (MTBE) containing 0.1% (w/v) butylated hydroxytoluene (BHT) and filtered through 0.22 mm nylon membrane. This filtered solution was used for detection of lycopene and β -carotene contents. The same carotenoid extraction method was used, when the dried carotenoid extracts were dissolved in 1 mL ethyl acetate containing 0.1% (w/v) BHT, the filtered solution was used to measure lutein, zeaxanthin and β -cryptoxanthin contents.

High performance liquid chromatography (HPLC) analyses of carotenoid extracts were performed on a Waters e2695 (Waters, Massachusetts, USA) unit equipped with a photodiode array detector (PAD). Lycopene and β -carotene were separated on an AcclaimTM C₃₀ column (4.6 × 250 mm, 5 μ m) and were gradient eluted with methanol/acetonitrile (1:3, v/v; eluent A) containing 0.1% (w/v) BHT and MTBE (eluent B) containing 0.1% (w/v) BHT. Lutein, zeaxanthin and β -cryptoxanthin were measured with another strategy. The three carotenoid components were separated using YMC Carotenoid C₃₀, 4.6 × 250 mm, 5 μ m (YMC, Wilmington, NC, USA) with solvent A (methanol/water, 97:3, v/v), and solvent B (100% MTBE). Lycopene, β -carotene, lutein, zeaxanthin and β -cryptoxanthin standards were purchased from Yuanye Company (Yuanye, Shanghai, China). Carotenoid components were quantified by both the retention times and absorption spectrum curves.

qRT-PCR validation

The CTAB protocol was used to extract total RNA from pericarp according to Zhu et al. (2008). Four microgram of total RNA was used for first stand cDNA synthesis by Prime-Script RT reagent Kit with gDNA Eraser (Perfect Real Time RR047A) (Takara, Dalian, China) following manufacturer's instructions. The cDNA was diluted 10-fold and used as template for qRT-PCR. Determination of the relative expression levels was performed on a Mastercycler ep realplex instrument (Eppendorf, Hamburg, Germany), using the SYBR Premix DimerEraser Kit (Perfect Real Time RR091A) (Takara, Dalian, China). The primers used in this study were designed using primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3>). The primer and the sequences were listed in Supplemental Information – Tables S2 and S3, respectively. The fold change of gene expression was calculated by using the formula $2^{-\Delta\Delta CT}$ and normalized to the actin gene of *Citrus maxima* (GenBank accession number: GQ389668.1).

Results

Measurement of carotenoid content

Carotenoid levels in the pericarp of ripe fruits from 'Huangbao' and 'Guanxi' were determined using high-performance liquid chromatography (HPLC). In 'Huangbao' pummelo, lycopene was the main contributor to carotenoid accumulation; lutein and zeaxanthin were not identified. Howev-

TABLE 1. Carotenoid contents in the pericarp of ripe fruits from 'Huangbao' and 'Guanxi' ($\mu\text{g g}^{-1}$ FW).

Cultivars	β -carotene	Lycopene	Lutein	Zeaxanthin	β -cryptoxanthin
Huangbao	0.74 $\pm$ 0.03	160.29 $\pm$ 22.60*	–	–	0.72 $\pm$ 0.21
Guanxi	0.70 $\pm$ 0.05	0.91 $\pm$ 0.18	0.42 $\pm$ 0.08	–	0.13 $\pm$ 0.13

–: Undetected. Data are mean $\pm$ SE ($n=3$). Data were analyzed by using ANOVA with Duncan's multiple range test.

*: Significant level was tested at $p \leq 0.05$.

er, minor amounts of carotenoids were detected in ‘Guanxi’ pummelo (Table 1).

RNA-sequencing and assembly of unigenes

Six cDNA libraries were created using ‘Huangbao’ and ‘Guanxi’ samples (i.e., each cultivar with three independent biological replicates). Clean reads were obtained by filtering the adaptor sequences and the low quality reads. The average GC content of clean reads was nearly the same (45%), and the average Q30 base percentage was more than 92%. Those high-quality reads were assembled *de novo* using the trinity method, generating 97377 unigenes with a mean length of 730 nt, and a N50 length of 1,188 nt. More than 78% of the clean reads per sample could be mapped to the assembled unigenes.

Functional annotation of unigenes

A total of 55,820 (57.32%) unigenes and 826 (88.91%) differentially expressed unigenes (DEGs) were annotated based on sequence alignment using eight public databases. A BLAST search was performed with the unigenes against the NR database with a threshold e-value of $< 1e^{-5}$. Most of the unigenes presented homology with the matched sequences with an e-value lower than $1e^{-15}$. Species specific distribution showed that 27.20% of the unigenes matched gene sequences of *Citrus sinensis*, followed by *Citrus clementina* (15.04%).

All unigenes were annotated within GO database into three main categories, including biological process (BP), molecular function (MF) and cellular component (CC) (Figure 2). In the BP category, the predominant subcategories were metabolic process, cellular process, response to stimulus, biological regulation, localization and signaling. In case of MF, most of the genes belonged to catalytic activity, binding, transport-

er activity, nucleic acid binding transcription factor activity, enzyme regulator activity, and protein binding transcription factor activity. As far as CC was concerned, the predominant subcategories were cell part, organelle, membrane, macromolecular complex, membrane part, membrane-enclosed lumen, cell junction and symplast. Functional annotation of unigenes in COG, KOG and eggNOG databases is shown in Supplemental Information – Figures S1–S3.

Global detection of DEGs

The comparison of transcriptomes of the two pummelo cultivars identified 929 DEGs showing a significant change in expression level (FDR < 0.01 and fold change ≥ 2), including 326 up-regulated genes and 603 down-regulated genes. The fold changes of the majority of those DEGs were distributed in the range of 2–10. It was observed that 130 and 150 DEGs specially expressed in the pericarp of ripe fruits from ‘Huangbao’ and ‘Guanxi’, respectively. Of the specially expressed DEGs, 90% exhibited a low transcript level (FPKM < 5).

GO enrichment analysis of DEGs

In BP category, “cell surface receptor signaling pathway”, “light harvesting”, “metabolic process”, “oxidation-reduction process”, “auxin transport”, and “transmembrane receptor protein serine/threonine kinase signaling pathway” were the significantly enriched subcategories. Additionally, almost all of the genes classified into “translation” and “isoprene biosynthetic process” were specially identified in the pericarp of ripe fruits from ‘Huangbao’. In the case of CC, “plastid” and “chloroplast” were the most significantly enriched subcategories. Most genes classified into the “ribosome”, “plastoglobuli”, and “cytosolic small ribosomal subunit” subcate-

FIGURE 2. All unigenes were annotated within GO database into three main categories: biological process, cellular component, and molecular function. The Y-axis indicates the number of unigenes while the X-axis indicates the main GO categories.

gories were specially expressed in the pericarp of ripe fruits from 'Huangbao'. As far as the MF was concerned, "catalytic activity" and "DNA binding" were the highest represented subcategories, while "carotenoid isomerase activity", "metal ion binding", and "hydroxymethylglutaryl-CoA reductase activity" were also the significantly enriched groups.

KEGG pathway enrichment analysis of DEGs

A sum of 211 out of 929 DEGs were enriched in 89 KEGG pathways, among which, ten pathways were significantly enriched ($P < 0.05$) (Supplemental Information – Table S1). "Ribosome" contained the highest number of DEGs, in which most of those genes encoding for 60S ribosomal proteins and 40S ribosomal proteins specifically expressed in the pericarp of ripe fruits from 'Huangbao' pummelo. Followed by "carbon fixation in photosynthetic organism pathway", genes enriched in this pathway were mainly annotated as glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, and ribulose biphosphate carboxylase small chain.

DEGs involved in carotenoid and isoprene precursor metabolisms

Phytoene synthase and polycopene isomerase are the two enzymes involved in the upstream regulation of the lycopene biosynthesis. In this study, expression levels of genes encoding for the two enzymes were significantly lower in the pericarp of ripe fruits from 'Huangbao' as compared with 'Guanxi'.

We identified an up-regulated gene (c79299.graph_c0) encoding for fructose-bisphosphate aldolase, while c58950.graph_c0 and c49982.graph_c0, were two genes encoding for an isozyme of fructose-bisphosphate aldolase, that were specially expressed in 'Huangbao' and 'Guanxi', respectively. Fructose-bisphosphate aldolase catalyzes the formation of glyceraldehyde-3-phosphate. An up-regulated gene (c50907.graph_c0) was annotated as enolase and specially expressed in 'Huangbao'. Enolase catalyzes the conversion of 2-diphosphoglycerate to phosphoenolpyruvate. Three genes (c58669.graph_c0, c70874.graph_c0, c55863.graph_c0) were identified for pyruvate kinase isozyme and highly expressed in 'Huangbao' when compared to 'Guanxi'. Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate. Glyceraldehyde-3-phosphate and pyruvate are the precursors for IPP and DMAPP synthesis, while IPP and DMAPP can be used for carotenoid biosynthesis.

Three genes, c36907.graph_c0, c54686.graph_c0 and c38780.graph_c0 were down-regulated in 'Huangbao' and annotated as acetyl-CoA acetyltransferase, hydroxymethylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-coenzyme A reductase, respectively. The three corresponding enzymes are involved in MVA pathway. Transcript levels of the three genes indicated that MVA pathway activity in 'Huangbao' was lower than that in 'Guanxi'.

DEGs participated in phytohormone metabolic pathways

In the present study, several genes involved in gibberellin, cytokinin and ethylene metabolic pathways were differentially expressed between 'Huangbao' and 'Guanxi' cultivars. Genes encoding for gibberellin 2- β -dioxygenase (GA2OX) and glycine-rich RNA-binding protein were specifically expressed in 'Huangbao' and 'Guanxi', respectively. The cytokinin dehydrogenase gene was down-regulated in 'Huangbao'. A gene annotated as 1-aminocyclopropane-1-carboxylate synthase (ACS) was also down-regulated in 'Huangbao'. How-

ever, the transcript level of one gene encoding for 1-aminocyclopropane-1-carboxylate oxidase (ACO) was 25.69-fold higher in 'Huangbao' than that in 'Guanxi'.

DEGs associated with signal transduction

DEGs related to auxin and ethylene responsive proteins were down-regulated in 'Huangbao'. Four genes were identified for auxin responsive proteins, and one of them was annotated as putative indole-3-acetic acid-amido synthetase. In plants, this protein plays an important role in balancing active and inactive auxin contents by catalyzing the synthesis of indole-3-acetic acid-amido. Two genes (c67065.graph_c0b, c69145.graph_c0) were not identified in 'Huangbao'. The c67065.graph_c0b gene was annotated as EIN3-binding F-box protein 1 (EBF1), a negative factor in ethylene signaling pathway, while c69145.graph_c0 was a gene encoding ethylene insensitive 3 (EIN3), a positive factor in ethylene signal transduction.

Differentially expressed transcription factors and genes related to post-translational modification

Some transcription factors related to fruit ripening process, phytohormone signal transduction, pigment metabolism and terpene biosynthesis were down-regulated in 'Huangbao', including ethylene response transcription factor, MYB, DELLA, MADS-box and WRKY. However, most of the NAC domain-containing protein genes were up-regulated. Genes encoding for histone acetyltransferase, Ubiquitin-conjugating enzyme, glutathione S-transferase, and monothiol glutaredoxin were classified into "post-translational modification". They mainly participated in the biological processes of acetyl transfer, protein degradation, detoxication, and antioxidation. In which, histone acetyltransferase gene was highly expressed and was significantly up-regulated in 'Huangbao' pummelo.

DEGs related to transporter

Nine genes were observed to be encoding for ABC family members. In which, the gene (c73332.graph_c0) annotated as ABC transporter B family member 19 was not detected in 'Huangbao'.

Validation of DEGs by quantitative real time reverse transcription PCR (qRT-PCR)

Eight genes were randomly selected and used to verify the stabilization of the transcriptome analysis results. A qRT-PCR assay revealed that the relative expressions of the tested genes were coincident with the transcriptome profiles (Figure 3).

Discussion

Genes involved in carotenoid and its precursor metabolisms are differentially expressed in 'Huangbao' and 'Guanxi' pummelo cultivars

The transcript levels of the genes annotated as PSY and CRTISO were distinctly lower in 'Huangbao' than that in 'Guanxi', apparently, which was not coincident with the lycopene accumulation in 'Huangbao'. However, it was observed that the transcript abundance of PSY was not always coincident with the lycopene accumulation in tomato cultivar; its expression level at the fruit ripening stage decreased four-fold when compared with the colour break stage (Smita et al., 2013). Additionally, it has been reported that amino acid sequences influenced the activities of PSY and CRTISO (Gady

FIGURE 3. Verification of RNA-sequence data by qRT-PCR. Relative expression of genes was obtained from qRT-PCR, while FPKM was determined by RNA-sequencing method. Standard error bars indicate include three biological replicates.

et al., 2012; Fu et al., 2014; Li et al., 2010). These reports suggest that the post-transcription regulation of the two genes in lycopene synthesis should not be ignored. It has been found that down-regulation of β -*LCY* and capsanthin/capsorubin synthase gene were involved in the downstream metabolism of carotenoid biosynthetic pathway, which contributed to lycopene accumulation in the pulp of 'Hong Anliu' sweet orange fruits (Xu et al., 2009). Comparison of the findings in fruits from sweet orange and pummelo indicates that the regulatory mechanism involved in lycopene accumulation is various in different fruit types.

Glyceraldehyde-3-phosphate and pyruvate are the precursors for IPP and DMAPP synthesized through MEP pathway (Sponsel, 2002). Fructose-1, 6-phosphate conversion to glyceraldehyde-3-phosphate is catalyzed by fructose-bisphosphate aldolase. Enolase catalyzes the formation of phosphoenolpyruvate, and then, pyruvate kinase converts phosphoenolpyruvate to pyruvate. The expression levels of genes encoding for the three enzymes, were significantly higher in 'Huangbao' than that in 'Guanxi', which indicated that the biosyntheses of the original precursors for carotenoids may be promoted in 'Huangbao'.

Genes involved in other metabolic pathways are possibly associated with carotenoid accumulation in the pericarp of pummelo fruits

1. Genes implicated in phytohormone pathways. Carotenoid accumulation was accompanied by a decrease in GA content in tomtao fruits during ripening stage (Fraser et al., 1995). The application of exogenous GA led to increases in chlorophyll contents and decreases in carotenoid levels in the pericarp of lemon fruits (Borthakur and Ranjit, 2005). Therefore, gibberellins are not likely to facilitate carotenoid accumulation in fruits. In this study, *GA2OX* and *GRP* were specially expressed in 'Huangbao' and 'Guanxi', respectively. Active GA were decreased by *GA2OX* reaction (Dijkstra et al., 2008). Overexpression of *GRP* caused a decrease in transcript

abundance of a gene encoding for the first-step enzyme in the GA biosynthetic pathway, accordingly, the contents of GA4 and intermediate products of GA decreased (Löhr et al., 2014). The results of this study indicated that the GA activity or the GA metabolism was inhibited in the pericarp of pummelo fruits at ripened stage.

It has been observed that ethylene was involved in improving nutritional quality and colour development in fruits (Barry and Giovannoni, 2007). Lower production of ethylene led to lower carotenoid content in tomato fruits, and correspondingly, the orange fruit could not turn to red even at the ripening stage (Gao et al., 2016). While exogenous ethylene application elevated carotenoid content in loquat fruits and accelerated fruit colour change (Alos et al., 2019). In current study, ten DEGs were identified in ethylene signaling pathway, one of them was *EBF1*, and the remaining were ethylene responsive transcription factors, including *EIN3* and some members of ERF and RAP2 subfamilies. All of them had low levels of expression in 'Huangbao' when compared with 'Guanxi'. Particularly, *EBF1* and *EIN3* were only detected in 'Guanxi'. In tomato fruits, *ERF* and *AP2* were both negatively associated with ethylene synthesis, however, had opposite effects on carotenoid accumulation (Lee et al., 2012; Chung et al., 2010). *EIN* is a positive regulator of the carotenoid biosynthesis pathway (Fu et al., 2017). *EIN3* could interact with *EBF2* promoter and directly elevate its expression level (Konishi and Yanagisawa, 2008). EBF belongs to the F-box protein family and acts as a component in SCF complex, which could degrade EIN3 protein (Zhu and Guo, 2008). Based on the above reports and the findings in this study, it seemed that *ERF* transcription factors in ethylene signal pathway were more critical for carotenoid accumulation in the pericarp of ripe pummelo fruits.

Application of indole-3-acetic acid (IAA) decreased transcript levels of several genes (*PSY1*, *PSY3*, *PDS*, *ZISO* and *CRTISO*) involved in the up-stream carotenoid metabolic pathway, but elevated the expression of down-stream genes (β -*LCY1*

and *CRTR-β1*). Consequently, the tomato fruit could not turn red due to a significant decrease in lycopene content and a remarkable increase in violaxanthin and neoxanthin contents (Su et al., 2015). It has been reported that ABC transporter B family member 19 (*ABCB19*) is capable of transporting auxin in plant (Cho et al., 2014). In the present study, *ABCB19* was only detected in ‘Guanxi’. Due to the fact that auxin plays a negative role in lycopene synthesis in fruits, *ABCB19* may be an important candidate gene involved in regulating carotenoid accumulation in pericarp of pummelo fruits.

2. Transcription factors. In pea (*Pisum sativum* L.), the DELLA protein down-regulated the expression of *PsGA2ox1* (a GA deactivation gene) (O'Neill et al., 2010). In this study, DELLA protein gene was specially expressed in the pericarp of ripe fruits from ‘Guanxi’. Therefore, it can be deduced that the GA activity is not effectively suppressed in ‘Guanxi’. These findings indicated that *GA2ox1* may act as an important regulator in carotenoid accumulation in pericarp of pummelo fruits.

In this study, some members of *NAC*, *MADS-box*, and *WRKY* transcription factor families were also differentially expressed in the two pummelo cultivars. Some *NAC* family genes promoted carotenoid accumulation, while others were negatively associated with carotenoid synthesis (Ma et al., 2014; Kou et al., 2018). Similarly, *MADS-box* gene family members functioned as a suppressor or an activator to regulate carotenoid metabolism (Yin et al., 2018; Dong et al., 2013). Further assays need to be conducted to define the roles of those *NAC* and *MADS-box* transcription factors in the modulation of carotenoid accumulation in pummelo fruits. *WRKY* transcription factor family is involved in response to stresses in plants (Xin et al., 2016). However, *WRKY* family genes are also involved in carotenoid metabolism. *OfWRKY3* could bind to the promoter region of *carotenoid cleavage dioxygenase 4* (*OfCCD4*) to transcriptionally activate its expression in sweet osmanthus (*Osmanthus fragrans*) (Han et al., 2016). *CCD4* is an enzyme catalyzing the cleavage of carotenoid substrates (Lashbrooke et al., 2013). Therefore, *WRKY* accelerate carotenoid degradation. The present study showed that *WRKY* was significantly down-regulated in ‘Huangbao’ as compared to ‘Guanxi’.

3. Genes associated with post translational modification. *SIHDT3* is a histone deacetylase and belongs to tomato HD2 family. When *SIHDT3* was silenced by RNAi technology, the amount of ethylene production and carotenoid accumulation decreased (Guo et al., 2017). *SIHDA3*, a member of tomato RPD3/HDA1 family of histone deacetylases, played a negative role in regulation of carotenoid accumulation in tomato fruits (Guo et al., 2018). Obviously, members in different histone deacetylase families play opposite roles in modulating carotenoid metabolism. In this study, expression level of the gene encoding for histone acetyltransferase was 36.95-fold higher in ‘Huangbao’ than that in ‘Guanxi’. The present study supports the idea that histone acetylation promotes carotenoid accumulation in fruits.

Conclusions

Lycopene is a major carotenoid component in the red-pericarp of ‘Huangbao’ fruits. Three genes (encoding for fructose-bisphosphate aldolase, enolase, and pyruvate kinase isozyme) involved in carbohydrate metabolism were significantly up-regulated in ‘Huangbao’, however, two genes (encoding for phytoene synthase and prolycopene isomerase) related to lycopene biosynthesis were significantly down-regulated. These results indicate that the regulation

of carotenoid metabolism maybe extended to the carbon skeleton biosynthetic pathway in the pericarp of pummelo fruits. *GA2OX* (inactivate gibberellin activity), *ABCB19* (auxin transporter) and *ERF* (ethylene signaling transduction) are the prominent candidate genes involved in the regulation of carotenoid metabolism in pericarp of pummelo fruits.

Acknowledgments

This work was supported by the Fujian Provincial Department of Science and Technology (Grant nrs. 2017R1013-3, 2018R1013-12, and 2020R10280014), and the public sharing platform for national scientific resources of the People's Republic of China (Grant nr. NHGRC2020-NH18-2).

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Received: Jan. 19, 2020

Accepted: Apr. 6, 2020

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL INFORMATION – TABLE S1. Unigenes involved in KEGG enriched pathways. Corrected P-value was calculated by adjusting P-value using the Bonferroni approach.

KEGG pathway	ko ID	Number of DEGs in pathway	Number of all genes in pathway	P-value	Corrected P-value
Ribosome	ko03010	39	1055	6.67E-07	5.94E-05
Flavonoid biosynthesis	ko00941	9	122	1.52E-04	0.01
Photosynthesis - antenna proteins	ko00196	8	98	1.76E-04	0.02
Circadian rhythm - plant	ko04712	7	147	9.55E-03	0.85
Photosynthesis	ko00195	9	224	0.01	0.91
ABC transporters	ko02010	5	84	0.01	1
Ascorbate and aldarate metabolism	ko00053	7	169	0.02	1
Carbon fixation in photosynthetic organisms	ko00710	11	355	0.03	1
Cutin, suberine and wax biosynthesis	ko00073	3	42	0.03	1
Fatty acid elongation	ko00062	4	76	0.03	1
Cysteine and methionine metabolism	ko00270	10	344	0.05	1
Arachidonic acid metabolism	ko00590	3	53	0.05	1
Stilbenoid, diarylheptanoid and gingerol biosynthesis	ko00945	3	55	0.06	1
Taurine and hypotaurine metabolism	ko00430	2	29	0.08	1
Synthesis and degradation of ketone bodies	ko00072	2	29	0.08	1
Glycolysis / Gluconeogenesis	ko00010	12	493	0.10	1
Nitrogen metabolism	ko00910	4	110	0.10	1
Biosynthesis of amino acids	ko01230	20	941	0.12	1
Phenylalanine metabolism	ko00360	6	233	0.17	1
Brassinosteroid biosynthesis	ko00905	1	13	0.19	1
Porphyrin and chlorophyll metabolism	ko00860	4	141	0.19	1
Thiamine metabolism	ko00730	2	51	0.20	1
Phenylpropanoid biosynthesis	ko00940	7	298	0.20	1
Zeatin biosynthesis	ko00908	1	15	0.22	1
Phenylalanine, tyrosine and tryptophan biosynthesis	ko00400	4	152	0.23	1
Linoleic acid metabolism	ko00591	2	62	0.26	1
Terpenoid backbone biosynthesis	ko00900	4	164	0.27	1
Biosynthesis of unsaturated fatty acids	ko01040	3	116	0.29	1
Pentose and glucuronate interconversions	ko00040	4	181	0.33	1
Diterpenoid biosynthesis	ko00904	1	25	0.33	1
Sesquiterpenoid and triterpenoid biosynthesis	ko00909	1	25	0.33	1
Amino sugar and nucleotide sugar metabolism	ko00520	7	352	0.34	1
Cyanoamino acid metabolism	ko00460	3	128	0.34	1
Carbon metabolism	ko01200	19	1071	0.36	1
Valine, leucine and isoleucine degradation	ko00280	4	197	0.39	1
Carotenoid biosynthesis	ko00906	2	91	0.43	1
Degradation of aromatic compounds	ko01220	1	35	0.43	1
Tropane, piperidine and pyridine alkaloid biosynthesis	ko00960	2	92	0.44	1
Pentose phosphate pathway	ko00030	4	211	0.44	1
Fructose and mannose metabolism	ko00051	4	214	0.45	1
Butanoate metabolism	ko00650	2	95	0.45	1
Fatty acid metabolism	ko01212	5	277	0.46	1
Vitamin B6 metabolism	ko00750	1	43	0.50	1
Sulphur metabolism	ko00920	2	111	0.53	1
Tryptophan metabolism	ko00380	2	112	0.54	1
Plant hormone signal transduction	ko04075	8	514	0.59	1
Folate biosynthesis	ko00790	1	56	0.60	1
Galactose metabolism	ko00052	3	196	0.61	1

SUPPLEMENTAL INFORMATION – TABLE S1. Continued.

KEGG pathway	ko ID	Number of DEGs in pathway	Number of all genes in pathway	P-value	Corrected P-value
Phosphatidylinositol signaling system	ko04070	2	134	0.64	1
Glutathione metabolism	ko00480	4	272	0.64	1
Glyoxylate and dicarboxylate metabolism	ko00630	4	275	0.65	1
Arginine and proline metabolism	ko00330	4	280	0.66	1
Ether lipid metabolism	ko00565	1	68	0.67	1
Ubiquinone and other terpenoid-quinone biosynthesis	ko00130	2	144	0.68	1
One carbon pool by folate	ko00670	1	72	0.69	1
RNA degradation	ko03018	5	360	0.69	1
Alpha-Linolenic acid metabolism	ko00592	2	149	0.69	1
Plant-pathogen interaction	ko04626	6	432	0.70	1
Isoquinoline alkaloid biosynthesis	ko00950	1	76	0.71	1
SNARE interactions in vesicular transport	ko04130	1	78	0.72	1
Pyruvate metabolism	ko00620	5	382	0.74	1
Valine, leucine and isoleucine biosynthesis	ko00290	1	83	0.74	1
Steroid biosynthesis	ko00100	1	85	0.75	1
Pantothenate and CoA biosynthesis	ko00770	1	88	0.76	1
Inositol phosphate metabolism	ko00562	2	170	0.76	1
Fatty acid degradation	ko00071	2	176	0.78	1
mRNA surveillance pathway	ko03015	4	340	0.80	1
Sphingolipid metabolism	ko00600	1	100	0.80	1
Starch and sucrose metabolism	ko00500	6	495	0.81	1
Selenocompound metabolism	ko00450	1	104	0.82	1
Glycerophospholipid metabolism	ko00564	2	193	0.82	1
Protein processing in endoplasmic reticulum	ko04141	9	718	0.82	1
Alanine, aspartate and glutamate metabolism	ko00250	2	206	0.85	1
Phagosome	ko04145	3	292	0.85	1
Lysine degradation	ko00310	1	120	0.86	1
Propanoate metabolism	ko00640	1	129	0.88	1
Protein export	ko03060	1	131	0.88	1
Purine metabolism	ko00230	5	478	0.89	1
Tyrosine metabolism	ko00350	1	135	0.89	1
Endocytosis	ko04144	3	359	0.93	1
Fatty acid biosynthesis	ko00061	1	164	0.93	1
RNA transport	ko03013	5	544	0.94	1
Peroxisome	ko04146	2	287	0.95	1
2-oxocarboxylic acid metabolism	ko01210	1	206	0.97	1
Glycine, serine and threonine metabolism	ko00260	1	252	0.98	1
Ubiquitin mediated proteolysis	ko04120	2	384	0.99	1
Oxidative phosphorylation	ko00190	3	543	0.99	1
Pyrimidine metabolism	ko00240	1	320	0.99	1
Spliceosome	ko03040	3	627	1.00	1

SUPPLEMENTAL INFORMATION – TABLE S2. Primer used for qRT-PCR amplification and the validation of expression levels of DEGs, related to Figure 3.

Gene ID	Forward primer (5'-3')	Reverse primer (5'-3')	Functional annotation
c39673.graph_c0	5' CGGGACAAGAAGAGGAATTG 3'	5' AGAAGAAGCTCCGCAATGAA 3'	Trans-cinnamate 4-monooxygenase
c64297.graph_c0	5' AAGGATGAACAGGACACAAGC 3'	5' TCGATATTCTGGATCGCTCA 3'	Chalcone synthase 2
c69613.graph_c0	5' TCCGATGCCCTCTACCAAGTAT 3'	5' TTCAAGAACTGCCCTTCGTC 3'	Caffeoyl-CoA O-methyltransferase 1
c84089.graph_c0	5' GCCTAGCCAAACCAGGAGTA 3'	5' TGAGTGTGAGGTGGGTTTCA 3'	Superoxide dismutase activity
c83333.graph_c0	5' ACACCGCTTTGACTTCGTCT 3'	5' TTCTCCGTGGATCAATTCT 3'	ABC transporter I family member 20
c79426.graph_c0	5' GCAAAGCCAGCTCAAAACT 3'	5' ACTATCTTCCAGCCGGAAT 3'	ABC transporter B family member 2
c83287.graph_c0	5' CATTTTCCTGCCACTTCGAT 3'	5' CTTCGGTGTGTTGGTCTTCA 3'	3-ketoacyl-CoA synthase 10
c83745.graph_c0	5' ATCTCACCAAGCCAGAAGA 3'	5' CCCAGGTTTGTACCTTGCTT 3'	Very-long-chain (3R)-3-hydroxy-acyl-CoA dehydratase
GQ389668.1 (endogenous control gene)	5' CTGAGCGGGAAATTGTTAGG 3'	5' GCCCAATAGTGATGACCTG 3'	Actin (<i>Citrus maxima</i>)

SUPPLEMENTAL INFORMATION – TABLE S3: See www.ishs.org/eJHS. **SUPPLEMENTAL INFORMATION – FIGURE S1.** Functional classification of unigenes annotated in the COG database. The Y-axis represents the number of annotated unigenes, and the X-axis represents the functional classification.

SUPPLEMENTAL INFORMATION – FIGURE S2. Functional classification of unigenes annotated in the KOG database. The Y-axis indicates the number of annotated unigenes, and the X-axis indicates the functional classification.

SUPPLEMENTAL INFORMATION – FIGURE S3. Functional classification of unigenes annotated in the eggNOG database. The Y-axis shows the number of annotated unigenes, and the X-axis shows the functional classification.