Full Length Article

Comprehensive Transcriptome Analyses of Carbohydrate Metabolism and Flavonoid Biosynthesis in Blueberry (*Vaccinium corymbosum*) during Fruit Maturation

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Abstract

In blueberry, the coordinated changes in carbohydrates and anthocyanins are the most obvious aspects of the complex process in fruit maturation, containing flavor and nutritional transformation, such as sweetness and color changes. After years of artificial breeding, the taste quality and active ingredient content of blueberries have been significantly improved. However, our knowledge of the relevant mechanisms of gene expression and metabolic pathways is still limited. In our study, RNA sequencing analysis was performed on the fruits of two varieties at green, pink and blue fruit stages (42, 49 and 56 days after flowering). About 40.54 Gb of clean reads and 109,480 unigenes were obtained. All of 48 and 18 DEGs related to carbohydrate metabolism and anthocyanin synthesis were identified. We suggest that SPS and NI played important role for fructose and glucose accumulation. Citrate degradation was associated with CS and ME in the acetyl-CoA pathway, and with IDH, GABA-AT, GAD and GS in the GABA shunt. F3′H, F3′5′H, FLS and UFGT regulated flavonoid biosynthesis. Our data suggest that sugar and acid metabolism and flavonoids synthesis in blueberry fruits are regulated by development and genotype. This study systematically analyzed the genes related to carbohydrates metabolism and flavonoids biosynthesis in blueberry, providing a foundation for research of these genes function and regulation of related fruit quality traits. © 2020 Friends Science Publishers

Introduction

Carbohydrate is one of the most important products of plant photosynthesis, which not only participates in plant morphogenesis, but also serves as an important energy supply during plant growth and development (Raessler *et al.* 2010). Especially, as an important indicator of plant carbon absorption and consumption, sugar is both photosynthetic product and respiratory substrates, which provide carbon skeletons and energy for plants. Sugar is also a precursor for the synthesis of many plant secondary metabolites, such as phenols, defense and aromatic compounds (Li *et al.* 2020).

Furthermore, sugar is also believed to be a signaling molecule that regulates plant development and defense responses (Ruan *et al.* 2010; Cho and Yoo 2011). Therefore, the synthesis and metabolism of carbohydrates, especially sugars, affect the whole process of plant growth and development. Sugar accumulation is critical to crop yield and quality formation, and appropriate sugar-acid ratio is also the core of fruit commercial quality in fruit trees.

Highbush blueberry, the shrub of Ericaceae family, is a kind of the most famous commercial berry in the world (Kim *et al.* 2013). For the past few years, blueberries have received a lot of attention for their powerful antioxidant properties and abundant active ingredient, such as high levels of anthocyanins, flavonols and proanthocyanidins (Prior *et al.* 2001; Castrejó *et al.* 2008; Vrhovsek *et al.* 2012; Gibson *et al.* 2013). In blueberry, the coordinated changes in carbohydrates and anthocyanins are the most obvious aspects of the complex process in fruit maturation, containing flavor and nutritional transformation, such as sweetness and color changes (Zifkin *et al.* 2012). The fruit ripening is accompanied by rapid changes in color, taste, flavor, and nutrients in blueberry, which caused by the accumulation of large amounts of certain metabolites such as sugar, organic acids, phenolic compound, and especially anthocyanins (Rasmussen *et al.* 2005). The contents of these metabolites vary over time, which is attributed to the different rates of synthesis, degradation and transportation during fruit ripening. The degree of sugar accumulation largely determines the sweetness of the fruit at harvest (Kader 2008). The TCA cycle and GABA shut also determines the fruit acidity (Liu *et al.* 2007; Zhang *et al.* 2014), and flavonoid accumulation not only makes the fruit appear blue, but also is the main reason for the formation of antioxidant activity in blueberry (Zifkin *et al.* 2012; Harb *et al.* 2010). Carbohydrate metabolism and flavonoids synthesis have been the focus of fruit quality research. However, the metabolism and accumulation of such substances is a complex physiological process, the mechanism of carbohydrate metabolism and flavonoid synthesis and key enzyme genes have not yet been identified in blueberry fruit maturation.

Blueberry ploidy is highly heterozygous and has a large genome (1216 Mbp) size (Costich *et al.* 1993; Bian *et al.* 2014; Colle *et al.* 2019). The short of information about blueberry genome sequences affects our understanding of molecular information about key quality changes during ripening. High throughput omics provides reliable way for studying the complex regulatory processes about metabolic pathways, gene expression or gene regulatory networks (Wang *et al.* 2017). Currently, transcriptome and multiple omics studies based on high throughput sequencing have been applied to research growth and development and the physiological synthesis of active substances in blueberry, but mainly focused on the study of plant cooling capacity (Naik *et al.* 2007; Dhanaraj *et al.* 2007), flower bud differentiation (Albert *et al.* 2005), and antioxidant

metabolism of fruit polyphenols (Li *et al.* 2012; Lin *et al.* 2018; Li *et al.* 2019). There are no reports on the mechanism of fruit quality formation, most of related enzyme genes have not been identified. The gene expression pattern at different developmental stages and genotypes need to be further verified.

In order to confirm the optimal harvest time, obtain better taste and more nutritious varieties, we identified key enzyme genes encoding carbohydrate metabolism and accumulation of flavonoids biosynthesis based on transcriptome sequencing and gene expression analysis of different two varieties at three development period. We tentatively speculated the role of these genes in blueberry fruit maturation. The purpose of this research was to explore the main mechanism of fruit quality formation in blueberry.

Materials and Methods

Plant material

The blueberry fruits (*Vaccinium corymbosum* L. cvs. 'Spartan' and 'Raka') used in this study were collected from an greenhouse in Liaoning Institute of Pomology, XiongYue, Liaoning Province, China. Five trees of 5-year-old from each cultivar were chosen to exclude stochastic error caused by different genetic and environment backgrounds. Fruit of three different stages, including green, pink and blue fruit stage (42, 49 and 56 days after full bloom), were gathered from ten different trees, denoted as S1, S2, S3, R1, R2, R3 (Fig. 1). All fruits were flash-frozen in liquid nitrogen and stored in ultra-low temperature freezer at -80℃ after were transported to the laboratory.

Soluble sugar content (SSC) and titratable acid (TA) measurement

The mixed juice extracted from ten fruit in same developmental period to measure SSC using PAL-LOOP (Atago Japan). The SSC of the fruit was represented by the mean of three replications. TA was measured using NaOH titration according to method of Shiraishi (1995).

Total anthocyanin content (TAC) and total phenols content (TPC) measurement

The extraction of TAC and TPC followed the modified methods of Kim *et al.* (2013). Ten frozen fruits of each sample were mixed and ground into powder in liquid nitrogen. Then 5 g powder was added to ethanol, distilled water and hydrochloric acid solution (70:30:1, v/v/v), and centrifuged for 20 min. After repeat centrifugation for twice, combined solution, and set the total volume to 20 mL using for testing.

The TAC was measured using pH differentia method (Connor *et al.* 2002). The extracting solution was diluted by acidic methanol (1:99, v/v), the absorbance value was

determined at 530 nm. The standard curve was established with cyanidin-3-glucoside as the standard substance, and the total anthocyanin content was expressed as mg/100g standard substance equivalents.

The TPC was measured using Folin-Ciocalteu method (Pastrana-Bonilla *et al.* 2003). The extracting solution (0.04 mL) was added Folin-Ciocalteu (1 mL), 75% sodium carbonate (0.8 mL) and distilled water (0.16 mL). After mixed and incubated for 30 min, the absorbance value was determined at 765 nm. The standard curve was established with gallic acid as the standard substance, and the total phenols content was expressed as mg/100g standard substance equivalents.

RNA extraction, cDNA library construction and RNA-seq

Total RNA was extracted using the modified CTAB method (Jaakola *et al.* 2001). After treated using DNase I, the samples were enriched with magnetic beads and broken into mRNA short fragments. These short fragments were reverse transcribed into cDNA, and proceeded with the purification of double-stranded, terminal repair, addition of A tail and connection. About 200–700 bp fragments were separated and used as templates for PCR amplification to obtain the cDNA library. Six libraries were tested the sequence from double-ended on the Illumina HiSeq™2000 platform.

Sequence assembly and annotation

The original image data file from RNA-Seq via CASAVA bases to identify analysis into the original data (raw data). After detecting the error rate, ATGC distribution, and filtering the reads containing adapter, ploy-N and low quality from raw data, we finally obtained the clean reads. The subsequent analysis was based on clean reads. Trinity was used to transcriptome assembly with min_kmer_cov set as two and all of other arguments set as default (Grabherr *et al.* 2011). The sequences of six samples were mixed and spliced to serve as genome reference sequences.

Based on the NR, NT, Swiss-Prot, Pfam, gene ontology (GO), KOG, and KEGG Ortholog databases (KO), function annotation information of genes were obtained. The amino acid sequences and the coding regions information of genes was predicted using BLAST. ESTScan software was used to predicted others sequence information, when its none annotated above databases (Iseli *et al.* 1999).

Differential expression analysis

Clear reads of each sample were mapped back onto the reference sequence to calculate the read-count (Li and Dewey 2011). Considering the impact of sequencing depth and gene length, we transformed readcount into RPKM (Mortazavi *et al.* 2008) to expression the relative expression levels. DEGs of two samples were identified using the DEGseq software. To control false positives, *p*-value and log$_2$ (fold change) were used to screen the DEGs. In this study, DEGs need to be matched *q*-value ≤0.005 and |log$_2$ (fold change)| ≥1, *q*-value was adjusted using q value (Storey 2003).

Functional enrichment analysis

To comprehend the profile and function of DEGs that were obtained on different samples, we performed gene ontology analysis and conducted the functional enrichment using GOseq software (Young *et al.* 2010). In pathway analysis, all DEGs were mapped to the terms of KEGG. We used KOBAS (Mao *et al.* 2005) software to test the statistical enrichment of DEGs in KEGG pathway.

Quantitative real-time PCR (q-PCR)

Q-PCR reaction system (10 μL per volume) consisted of 2 μL of cDNA, 0.5 μL of each primer (Table 1), 5 μL SYBR Premix Ex TaqTM II, and 1.5 μL of RNase-free ddH$_2$O. The q-PCR was reacted on Real-Time System (Bio-Rad, USA). The thermal cycling conditions were set at 5 min at 95ºC, then followed by 30 cycles of 94ºC for 30 s, 58ºC for 30 s and 72ºC for 30 s. finally, q-PCR ended with a melting curve analysis program. Primers specificity was commanded by dissociation curves and PCR products. The mean Ct values were normalized to the acting gene (GAPDH, GenBank accession no. AY123769). The levels of gene expression were calculated using 2$^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001).

Statistical analysis

Physiological indicators were estimated in a completely randomized design with three replications. Statistical significance of differences was calculated using SPSS 19.0, and differences among the treatments were considered significant at *p*-value < 0.05.

Results

Changes in SSC, TA, TAC and TPC during fruit ripening

The changes of fruit color, sugar and acid are important characteristics of fruit maturation. At early stage, the skin color of fruit was green, the titratable acid content was higher, the SSC and TA content in the fruit between 'Spartan' and 'Rake' were almost equal, and the anthocyanin and total phenol contents were very low (Fig. 1; Table 2). As the fruit matured, the sugar and anthocyanin content in 'Spartan' fruit rapidly accumulated, while the sugar content in 'Rake' fruit was relatively slower, and titratable acid content showed an obvious decreasing trend. At this time, the TPC in the fruit significantly increased.

Table 1: The primers of qRT-PCR reaction

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	PCR products (bp)
114368_c1	AGCTGTTATGGCAGGTTTATGC	GGTGGATCTGGACTGGAAGG	137
97621_c0	CGATGCTAACGGTGTCTGGA	TCGGATAGGTTGGCTGGATA	129
99130_c0	CATTGCCGACTGCCTTGA	TTACCCACTGCACCGCTTA	147
108372_c0	GATGCACGCCGGATTAGC	CGTTTGTCTTTCCCACCCA	165
110081_c0	ACTTTGTCCCCACCGTCTCC	GCCAGCATCCTCCCGAAC	163
109295_c0	CAAAATCTTCCCTTTCACATCC	CGTCCTTGCCCAGTCCAT	179
107092_c0	GGTTTTGGAGACGAGGGG	AATTGATATGAACTTGCGATGC	104
110259_c0	GTTTCCCAACCGGAGTTCTTC	GCCTCAACCATACGCCAGTA	141
108040_c0	ACGAGATCCACGGCGACA	CAGCAATGAACAAGCGAAGC	172
107243_c0	GCTATTCCCTACTCGCTCACA	CCTAGCCAGTCACTGCCTTTC	126
114171_c2	AGGTTCGGTTGGATTGTGC	TCGTTGCTATCCTCATTCTCG	200
105148_c0	ACTCGGAAGGCCAAATAAGAA	CAGTAGTGAGGGCAGTCGGT	156
GAPDH	TGTTGTGGGAGTCAATGAGAAA	TGCGGTGATAGAGTGGATGG	157

Table 2: Variations of physiological characteristic in blueberry during fruit maturation

Sample	Soluble solid content (%)	Titratable acidity (%)	Total anthocyanin (mg.100 g^{-1})	Total polyphenol (mg.100 g^{-1})
S1	3.30±0.01f	1.32±0.01b	21.95±0.05e	335.16±0.03a
S2	7.80±0.00c	1.16±0.00c	87.19±0.08d	214.97±0.01e
S3	14.33±0.03a	0.35±0.06f	241.17±0.08b	283.26±1.00b
R1	4.17±0.03e	1.58±0.01a	17.94±0.01f	249.86±0.02d
R2	6.43±0.00d	0.91±0.01d	101.94±0.02c	173.73±0.03f
R3	8.17±0.03b	0.36±0.00e	299.20±0.08a	257.28±0.09c

RNA-seq and *de novo* assembly

To ascertain metabolic and gene expression changes during fruit ripening of different genotypes, transcriptome analysis was performed. Six libraries prepared of two varieties at three different development stages (Fig. 1). All of 40.54 Gb clean reads were acquired from RNA-Seq after removing the reads with adaptors and low quality reads (Table S1). Total of 109,480 unigenes and 223,382 transcripts were received, that N50s were 1111 and 1604, respectively. This showed a high quality assembly. Moreover, the total nucleotides of the unigenes and transcripts were 74,760,274 and 214,156,807. The mean lengths of the unigenes and transcripts were 683 and 959. Among the unigenes, 68,760 (62.81%) unigenes had lengths of 200–500 nt, 20,635 (18.85%) unigenes had lengths of 500–1000 nt, 12,817 (11.71%) unigenes had lengths ranging from 1000 to 2000 nt, and 7,268 (6.63%) had lengths of over than 2000 nt, to a mean length of 683 (Table 3).

Functional annotation and classification

To obtained the possible functional information of all unigenes, a work of sequence similarity search was executed in the NR, NT, KO, Swiss-Prot, Pfam, GO and KOG databases. The results showed that 30,721 (28.05%), 18,340 (16.75%), 10,758 (9.86%), 22,528 (20.57%), 25,061 (22.89%), 25,414（23.21%）and 11,516 (10.51%) unigenes were annotated in turn in the above seven databases. In conclusion, 4,022 unigenes were annotated in all of seven databases, 37,880 unigenes were annotated in at least one databases, with a threshold of $1.0E^{-5}$ (Table S2).

Based on the NR annotation, 6,492 (21.30%) of the unigenes appeared priority matches with sequences of *Vitis vinifera*, and only 1,687 (5.5%), 1,447 (4.8%) and 1,432 (4.7%) of the unigenes appeared priority matches with sequences of *Coffea canephora*, *Theobroma cacao* and *Sesamum indicum*, respectively (Fig. S1).

Based on the GO annotation, 25,414 unigenes were classified into 56 functional units which pertained to three primary categories: biological process, cellular component, and molecular function. Under the category of biological process, cellular process (14,369 unigenes, 23.76%) and metabolic process (13,674 unigenes, 22.61%) dominated the primary proportion. For the category of cellular component, large numbers of unigenes were categorized as cell (8,042 unigenes, 19.79%) and cell part (8,040 unigenes, 19.78%). About the category of molecular function, binding (13,976 unigenes, 46.18%) and catalytic activity (11,575 unigenes, 38.25%) were two main classification (Fig. S2).

Based on the KOG database, 11,516 unigenes were classified into 26 different functional groups (Fig. S3). A lot of the unigenes were included in 'General function prediction only'. But only one ungene was clustered in 'Unamed protein' category.

Based on the KEGG database, all of 10,758 unigenes were mapped into 249 KEGG pathways (Fig. S4). This showed that three largest pathways were carbohydrate metabolism (998 unigenes), translation (897 unigenes) and folding, sorting and degradation (812 unigenes). Among those metabolism categories, carbohydrate metabolism represented the most predominant pathway, which have received most attention with biosynthesis of other secondary metabolites in connection with fruit taste, color and oxidation resistance. Those annotations of gene were beneficial in identifying key genes in fruit ripening, and providing useful references for quality breeding in blueberry.

DEG expression and function enrichment

To show the level of gene expression, the read-count of gene was calculated and normalized to RPKM. Gene differential expression analysis was performed between different varieties of one development stage, and different development stages of same variety. Comparing the two varieties, all of 2,021 DEGs were found, with 1,324 (703 up, 621 down), 756 (475 up, 281 down) and 741 (395 up, 346 down) in green fruit stage, pink fruit stage and blue fruit stage, respectively (Fig. 2a). Between three stages, 2,413 and 1,055 DEGs were found in 'Spartan' and 'Raka'. 794 up and 672 down DEGs were observed between blue to green fruit stages in 'Spartan', and 456 up and 390 down DEGs were observed between blue to green fruit stages in 'Raka' (Fig. 2b–d).

To further clarify these gene expression patterns, we analyzed samples from three developmental stages in two varieties. Overall, 69 up and 41 down DEGs in carbohydrate metabolic process (GO: 0005975), 45 up and 29 down DEGs in single-organism carbohydrate metabolic process (GO: 0044723), 71 up and 78 down DEGs in oxidation-reduction process (GO: 0055114) were observed between R1 and S1. 51 up and 27 down DEGs in oxidoreductase activity (GO: 0016491) was observed between R2 and S2. 52 up and 35 down u DEGs in oxidoreductase activity (GO: 0016491), 50 up and 33 down DEGs in oxidoreductase activity (GO: 0016491), oxidation-reduction process (GO: 0055114), and 7 up and 6 down DEGs in antioxidant activity (GO: 0016209) were observed between R3 and S3. During fruit development, a lot of DEGs in carbohydrate were accumulated and antioxidant activity was improved in pink stage. But most of DEGs in blue stages were down-regulated (Table S3).

The KEGG pathway enrichment performed in all the DEGs. Comparing the two varieties, phenylalanine metabolism was primary different pathway. For different development stages, phenylalanine metabolism, flavonoid biosynthesis, starch and sucrose metabolism and biosynthesis of amino acids were the most significant pathways between pink stages with green stages. At blue with pink and green stages, flavonoid biosynthesis, phenylalanine metabolism and starch and sucrose metabolism were primary different pathway. Furthermore, we detected 9 and 14 DEGs in flavonoid biosynthesis and starch and sucrose metabolism (Table S4).

Recognizing genes related to sugar and organic Acid

A total of 14 DEGs related to sucrose metabolism were identified by RNA-Seq (Table S5). By comparison of two varieties in three stages, we found that SPS (113824_c0) was up-regulated in R1/S1 and down-regulated in R3/S3. NI (99130_c0) was up-regulated in R2/S2, respectively. FK (102118_c0) were down-regulated in R2/S2 and R3/S3. SUT (109295_c0) was down-regulated in R1/S1. We found

Table 3: Length distribution of all transcripts and unigenes

Transcript length	Transcripts	unigenes
200–500bp	100528(45.01%)	68760(62.81%)
500–1000bp	50606(22.65%)	20635(18.85%)
1000–2000bp	44404(19.88%)	12817(11.71%)
>2000bp	27844(12.46%)	7268(6.63%)
Total	223382	109480
Mean length	959	683
N50	1604	1111
Total nucleotides	214156807	74760274

TD:11. 18　TD:14. 61　TD:16. 75　TD:9. 50　TD:13. 21　TD:15. 38
LD:9. 64　LD:12. 69　LD:15. 12　LD:7. 65　LD:12. 10　LD:13. 26

S1　　S2　　S3　　R1　　R2　　R3

Fig. 1: Different stages of 'Spartan' and 'Raka' fruit ripening. TD: fruit transverse diameter (mm); LD: fruit longitudinal diameter (mm)

most of genes during fruit ripening were up-regulated in S3/S1, but SPS (113824_c0) and NI (99130_c0) were down-regulated in R3/R2 and R3/R1 (Fig. 3a).

Genes of starch hydrolysis *amy* (108744_c0) and *malZ* (112107_c0) were up-regulated in R1/S1, R2/S2 and R3/S3, and were up-regulated during fruit ripening. Gene of starch synthesis glgB (112085_c0) was down regulated in S2/S1, and no significant difference in S3/S2 and S2/S1 (Fig. 3b). In glycolysis/gluconeogenesis pathway, most of genes were up-regulated between pink stages with green stages in two varieties, but no significant difference between blue stages with pink stages and blue stages with pink stages. PFK (102118_c0) was up-regulated in R3/S3, PGM (111749_c0) and PDC (106622_c1) were up-regulated in R2/S2. Obviously, PEPCK (111269_c0) was up-regulated and PEPC (110823_c0) was down-regulated during fruit ripening (Fig. 3c).

Most of genes in TCA cycle and GABA shut were up-regulated during fruit ripening, no significant difference between two varieties. At the same times, we pated attention to the genes CS (110259_c0), IDH (109088_c1) and ME (96550_c0) were up-regulated in R3/R2, but down-regulated in S3/S2. On the contrary, GAD (114171_c2) and GS (108803_c0) were down-regulated in R3/R2, but up-regulated in S3/S2. In addition, CS (110259_c0) was up-regulated and GS (108803_c0) was down-regulated in R3/S3 (Fig. 3d–e).

Recognizing genes related to flavonoid

All of the 18 genes differentially expressed unigenes were related to flavonoid biosynthesis. By comparison two varieties in three stages (Table S5), we found most of genes
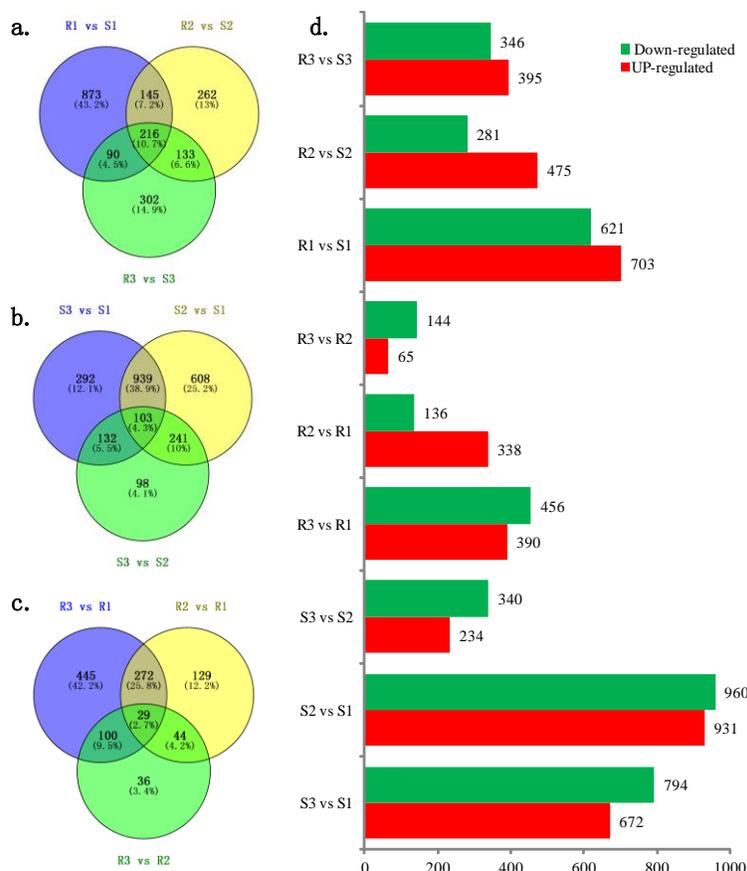
Fig. 2: Changes in gene expression profile among six samples. a–c. Vinn diagram showing the total number of DEGs between two cultivars of three stages. d. The numbers of up-regulated and down-regulated DEGs between each pair

were highly expressed in pink stages, but were little reduced at blue stages. Among them, F3′H (109742_0) and F3′5′H (111660_0) were up-regulated in R3/S3. PAL (100652_c1, 114146_c0 and 115162_c1), 4CL (111938_c0) and UFGT (111363_c0) were down-regulated in R1/S1. But DFR (102889_c0) and FLS (109531_c0) were up-regulated in R1/S1 and R2/S2 (Fig. 4).

Total of 1,165 Transcription factors, including 58 MYB and 65 bHLH, were Identified from 109,480 unigenes of RNA-Seq (Fig. S5). Heat map illustrating of transcription factors about MYB, bHLH and WD40 during blueberry fruit development was constructed (Fig. S6). We obtained 10, 8 and 9 unigenes of MYB, bHLH and WD40, respectively.

Verification of RNA-Seq results

To confirm the reliability of DEGs profiles that identified from transcriptome sequencing analysis. We selected 12 candidate DEGs to q-PCR assays, all of them had a high expression levels in most samples (Fig. 5). The results of q-PCR confirmed that the expression patterns of these DEGs were consistent with the transcriptome sequencing analysis results.

Discussion

Sweetness and antioxidant resistance were the key quality traits of blueberry fruit. Majority of fresh blueberry varieties on the market were north highbush blueberry. In this, some varieties showed lower sweetness and higher antioxidant activity, but the others showed higher sweetness and lower antioxidant activity. Today, we still poorly understood about the mechanism of sugars accumulation, organic acid metabolism and antioxidant formation in blueberry fruits.

In this experiment, we performed RNA-Seq to blueberry fruits at three developmental stages between two varieties, which indicated significant differences in SSC, TA, TAC and TPC between two varieties in maturity. Some genes and transcription factors related to sugars and organic acid metabolism and anthocyanin biosynthesis were found from transcriptome sequencing results. The analysis of the differential expressions of these genes in different varieties and stages of development showed that SPS and NI were key genes for sugar accumulation, ACO, NADP-IDH, GAD, GABA-AT and GS co-regulated organic acid metabolism, and F3'H, F3'5'H, FLS and UFGT were key enzymes regulating the biosynthesis of blueberry flavonoids.
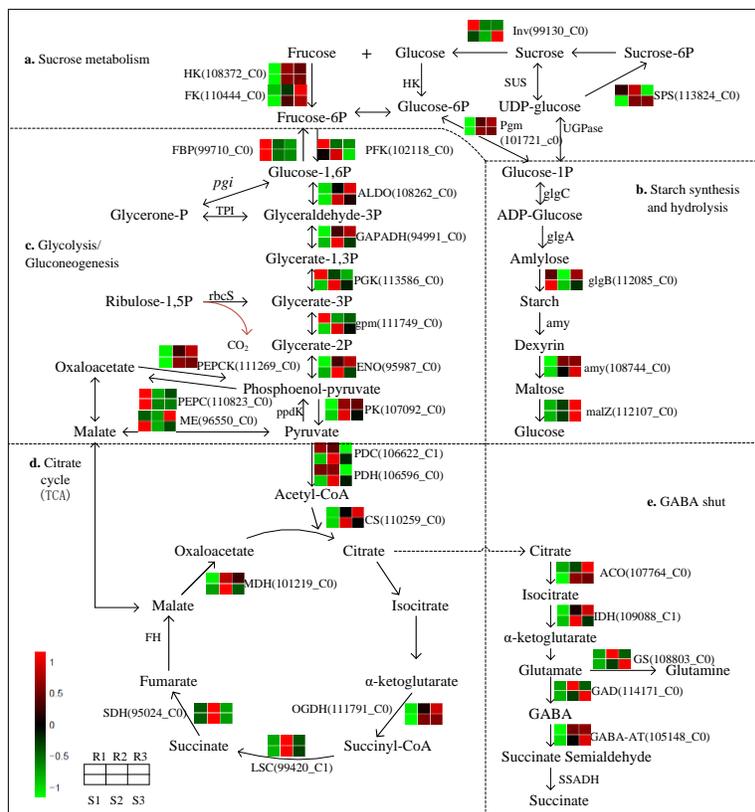
Fig. 3: Schematic of metabolic data related to blueberry fruit sugar and citrate during ripening
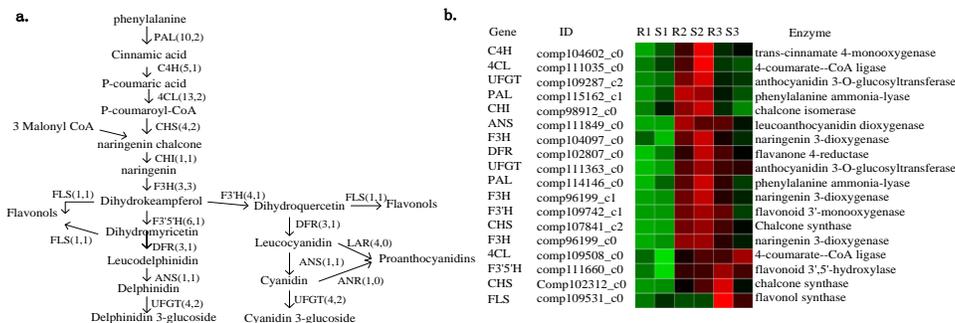

Fig. 4: Schematic of metabolic data related to blueberry fruit flavonoid during ripening. a. speculated pathway of flavonoid biosynthesis in blueberry. b. the pattern of gene expression

In most fruit trees, photosynthetic products are mainly sucrose, which is transported to fruit through phloem (Zhen *et al.* 2018). Sugars are constantly accumulated during fruit development and maturation. However, different fruit types had different patterns of sugar accumulation, and the effect of enzyme genes on sugar accumulation was also different. SPS dominates sucrose synthesis in strawberry, peach and kiwi fruit (Hubbard *et al.* 1991), especially, an increase in SS activity during fruit ripening played a major role in sucrose accumulation of peach fruit (Etienne *et al.* 2002; Desnoues *et al.* 2014). In addition, in apples (Tong *et al.* 2018), grapes (Xin *et al.* 2013), litchi (Yang *et al.* 2013),

pears (Vizzolo *et al.* 2003; Yao *et al.* 2010) and other fruits, hexose accumulation played a major role for fruit maturity. During fruit ripening, the activity of *Inv* increased and catalyzed sucrose to hydrolyze into fructose and glucose. Blueberry was a typical hexose accumulative fruit (Ayaz *et al.* 2001). Between two different varieties we tested, AI was up regulated throughout all ripening stages in both varieties, while NI and SPS were up regulated in the sweeter variety 'Spatan' and down regulated in 'Raka' at the fruit maturation stage. However, SS was not differentially expressed between two varieties in fruit maturation. We further speculated that SPS and NI express associated with
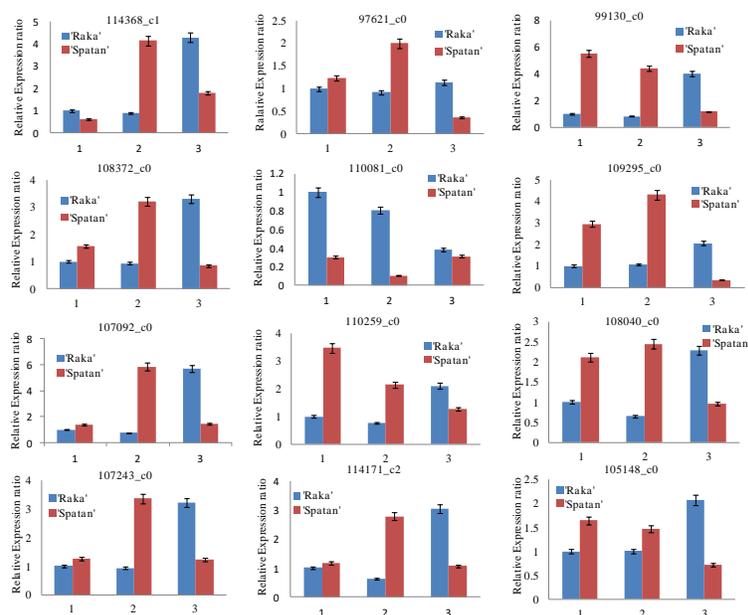
Fig. 5: qRT-PCR validation of 12 DEGs of 'Raka' and 'Spatan' during three fruit development stages

sugar accumulation during the ripening of the blueberry fruit. Other studies have shown that there was no correlation between Inv and sugar accumulation in grape (Davies and Robinson 1996). At the same time, both AI and NI regulated sugar accumulation in the same way in pear (Zhang *et al.* 2014). In addition, we also identified a SUT, which showed significant difference only in the two varieties in the green fruit stage, but no difference was noted at different stages of development. The specific relationship between this transporter and sugar accumulation needs verification.

The metabolic process of organic acid is very complicated in fruit. There are many reasons for the decrease of organic acid content in mature fruits, including increase in fruit volume, water content, greater decomposition of organic acid than synthesis, while organic acid plays a role in respiration and gluconeogenesis as a substrate (Sadka *et al.* 2003). In citrus (Cercos *et al.* 2006), citrate metabolism was connected with acetyl-CoA pathways and GABA shunt. PEPC and CS were main enzymes regulating the synthesis of citric acid. In the studies of melon and grape (Diakou *et al.* 2000; Tang *et al.* 2010) during different development stages, it was agreed that fruit malic acid accumulation was related to PEPC activity, while CS catalyzed oxaloacetic acid and acetyl-CoA to condense into citric acid, and acetyl-CoA was generated. During the development of loquat (Chen *et al.* 2009), changes in CS and PEPC were positively correlated with changes of TA content. However, other studies have suggested that there is no absolute correlation between CS activity and the levels of citric acid accumulation (Sadka *et al.* 2001). In addition, in the development of lemon (Sadka *et al.* 2003), the decrease of ACO activity in the mitochondrial promoted organic

acids accumulation in the early stage of fruit development, and the increase of ACO activity in the cytoplasm decreased the level of organic acids in the fruit maturation. At early stage of fruit development, NADP-IDH activity of mitochondria decreased, which promoted citric acid synthesis and inhibited ACO activity, leading to weakened TCA cycle. Therefore, it is believed that citric acid accumulation inhibited the activity of ACO and NADP-IDH in the TCA cycle. In this study, the PEPC activity of both varieties showed a downward trend with fruit ripening, and there was no significant difference between varieties. However, CS activity increased with fruit ripening in 'Raka', and slightly increased first and then decreased in 'Spatan'. It can be seen that the accumulation of citric acid in fruits is related to the activity of CS.

The expression pattern of NADP-IDH was the same with ACO in the two varieties. NADP-IDH was up regulated with fruit ripening in 'Raka', and slightly decreased with fruit ripening in 'Spatan'. In the GABA pathway, GAD, GABA-AT and GS were up-regulated with ripening in the fruit, up-regulated first in 'Raka' and then down-regulated in ripening fruit. Therefore, we believe that GABA pathway is the main pathway of citric acid metabolism in blueberry fruits, and ACO, NADP-IDH, GAD, GABA-AT and GS jointly regulated citric acid metabolism. In addition, ME played an important role in regulating the synthesis and degradation of fruit malic acid. We found that the expression of ME showed an opposite trend during the ripening process of the two blueberry varieties, rising gradually with fruit ripening in 'Raka' and decreasing gradually in 'Spatan'. In grapes, increased ME activity promoted malic acid production (Sweetman *et al.* 2009), while in apples, pears and plum, increased ME activity in the cytoplasm promoted

malic acid degradation (Berüter 2004; Mu *et al.* 2018). The specific role of ME in malic acid metabolism of blueberry remains to be further studied.

As the most widely studied pathway in plants, many structural genes and regulators in flavonoid biosynthesis have been cloned from *Arabidopsis thaliana*, *Zea mays*, petunia and other model plants (Lepiniec *et al.* 2006). In blueberry fruits, flavonoids mainly included flavonols, anthocyanins and procyanidins (Prior *et al.* 2001). Studies have shown that proanthocyanidins were mainly synthesized with a high expression in F3'H and a low expression in F3'5'H in the early stage of blueberry fruit development. When fruit matures, the concentration of proanthocyanidins decreases, and F3'H and F3'5'H increases (Zifkin *et al.* 2012). In apples, high concentration of PA was the result of high expression of F3'H gene and obvious deletion of F3'5'H gene (Han *et al.* 2010). The high concentration of PA was mainly related to the high expression of F3'5'H in persimmon (Akagi *et al.* 2009). In addition, proanthocyanidin biosynthetic genes ANR and LAR were expressed in early stage of fruit development, which were significantly separated from anthocyanin specific genes DFR, ANS and UFGT in time (Prior *et al.* 2001). In grape seeds, the expressions of F3'H, DFR and LAR were significantly increased at the initial stage of ripening, while ANR and ANS were also highly expressed (Bogs *et al.* 2005; 2006). However, this does not seem to be the case with blueberries. In this experiment, we found that the expression levels of F3'H and F3'5'H in the two varieties increased gradually during fruit ripening, and the expression of F3'H in the three stages of development was lower than F3'5'H. In the ripening fruits, the expression level of 'Raka' was significantly up regulated in 'Spatan'. However, there was no significant difference in the expression about proanthocyanidin synthesis genes ANR and LAR in different varieties and stages, which may be related to the decreased synthesis of proanthocyanidin in fruit ripening stage. In addition, FLS expression of flavonol synthesis gene was significantly higher than that of 'Spatan' in the pink fruit stage, and there was no significant difference between the two varieties in the late maturation stage. We speculated that flavonol and procyanidins were accumulated in the early stage of fruit development, and the concentration showed a decreasing trend as the fruit matured. In grapes and blueberries, anthocyanins synthesis occurs rapidly from the ripening stage, accompanied by rapid accumulation of UFGT in the peel (Jaakola *et al.* 2002). In this study, the expression levels of anthocyanin synthesis related genes DFR, ANS and UFGT were up regulated in both varieties, but there was no difference between varieties. In the whole flavonoids synthesis pathway, only CHS, F3'5'H and FLS were significantly higher than 'Spatan' in 'Raka' at maturity. Therefore, we hypothesized that the difference in flavonoid content between varieties was caused by the coordinated expression of the three genes. Studies have also shown that FLS of

flavonol synthesis gene and DFR of anthocyanin synthesis gene inhibit each other (Luo *et al.* 2016), which may be caused by the competitive substrates in the synthesis of different flavonoids, and the specific mechanism needs further experimental exploration.

Conclusion

To identify taste and oxidation resistance expression patterns during fruit ripening, a RNA-Seq data analysis between two varieties in three stages of fruit ripening was performed. We obtained the most related genes focusing on carbohydrate metabolism and flavonoid biosynthesis. Furthermore, gene expression and real-time quantification analysis access to the two key genes related to sugar accumulation, five key genes connected with organic acid metabolism and two key genes connected with anthocyanin accumulation. This indicated a strong involvement of anthocyanins in the fruit quality. This provided a new way to understand the molecular mechanisms underlying fruit quality formation in blueberry.

Acknowledgements

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