Differential expression of anthocyanin biosynthetic genes in relation to anthocyanin accumulation in the pericarp of mango (Mangifera indica Linn.)

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Abstract. We evaluated 14 mango cultivars with different pericarp color for pigments, chromatic parameters and mRNA level of anthocyanin synthesis gene. Pericarp of anthocyanin concentration varied from none to 15.96/100g FW among the 14 cultivars, which were divided into three coloration types, i.e. non-red (Deshehari, white ivory, Guire No.82, Guixiang, Lingshui, Qingpi and Yuexi No.1), medium type (Tainong No.1 and Chinhuang) and red (Renong No.2, Renong No.1, hongwacheng, Zillate, R2E2). From the results of expression of structure gene correlated with anthocyanin synthesis, the expression of the five genes, especially MiC4H, Mi4CL2, MiANS, MiDFR and MiUFGT2 in red cultivars were significantly higher than that of non-red cultivars.

1. Introduction

As the important tropical fruits, Mango (Mangifera indica L.) is popularly known as ‘The king of fruits’. China is one of the major mango producer, it has been cultivated nearly 140,000 ha in area and with an annual production of 1063,000 t. The plantation of mango has become the riching pillar industry of peasants in production area especially in youjiang river district and jinsha river dry-hot valley area.

The external color of fruit is an important factor in consumer preference. Skin colour of mango fruits refers to background colour and blush colour. The blush colour is the overlying orange, red or purple colour that formatted in the fruit development and it is often determined by the degree of exposure to direct sunlight [1]. Many cultivars in Southeast Asia lack or are poorly blushed, while the cultivars originated from Florida are highly coloured. In our institute, there has conserved 200 germplasm resources or cultivars and enriched pericarp colour, from green, yellow to pink, red and purple. Red mango cultivars (e.g.Tommy Atkins) are mainly the results of anthocyanin pigments, including cyanidin 3-O-galactoside [2] and 7-O-methylcyanidin 3-O-D-galactopyranoside[3].

The principal pigments are chlorophylls, carotenoids, and anthocyanins in mango fruit, which are synthesized via the terpenoid or phenylpropanoid pathways [4]. Anthocyanins have estimated from 203 to 565 mg/100 g DM depending on variety and stage of maturity [3], and the content of the ripe mango peel is higher than that of the raw peels.

Cloning of the structural genes in the anthocyanin biosynthetic pathway has been extensively reported in economically important fruit crops. The expression of the UFGT gene was critical for anthocyanin biosynthesis in the grape [5] and litchi [6]. In apple fruits, genes including CHS, F3H,
DFR, ANS and UFGT, are coordinately expressed during red coloration and their expression levels are positively related to anthocyanin concentration [7].

However, in mango, the information on molecular physiology of anthocyanin biosynthesis is quite limited. Only some data are available concerning anthocyanin content and related gene expression. Zhao et al cloned ANS, UFGT gene and studied the expression of ANS in three mango cultivars [8]. In this study, we selected fourteen structural genes anthocyanin biosynthetic enzymes, like PAL, 4CL, DFR, LDOX, ANS, UFGT1, UFGT2 based on NCBI data and transcriptome sequence in mango [9] and studied the expression of these genes in cultivars of different color types.

2. Materials and Methods

2.1. Plant material
Fruit samples of fourteen mango cultivars (Figure 1) including ‘Baixiangya’, ‘Guire No.82’, ‘Guixiang’, ‘Dashehari’, ‘Lingshui’, ‘Qingpi’, ‘Tainong No.1’ and ‘Chinhuan’, ‘Renong No.1’, ‘Renong No.2’, ‘R2E2’, ‘Hongwacheng’ and ‘Zillate’ were taken from Leizhou, Guangdong province, China. 10 fruits exposed to natural sunlight for each cultivar were picked randomly at green maturity. After color parameter measurements, pericarp were sampled immediately, and stored at -80°C for RNA extraction and other analyses.

2.2. Color analyses
The pericarp color were measured using a spectrum analyzer(Avaspec-2048-SPU) on 10 fruits samples immediately after picking. CIE L*, a*, and b* values were obtained and transformed into a hue angle degree (H = arc tan[b*/a*]) and chroma (C = [(a*)2 + (b*)2]1/2), which indicated the purity or intensity of the hue.

2.3. Determination of pigments
The total anthocyanin content was measured using the pH differential method and presented as mg cyanidin-3-galactoside per 100 g fresh tissue [10]. One gram of fruit peel was mixed with methanol containing 0.05% HCl, followed by centrifugation at 4°C and 12,000 rpm for 20 min. The absorbance of each 0.5ml extracted was assessed using a spectrophotometer at 525 nm and 700 nm in buffers of pH 1.0 and 5.0. Anthocyanin content was calculated using A = [(A525− A700) pH1.0 − (A525− A700)]
pH 5.0] with a molar extinction coefficient of cyanidin-3-galactoside of $3.02 \times 10^4$. Peel chlorophylls and carotenoids were determined according to Nagata and Yamashita [11].

### 2.4. RNA extraction and cDNA synthesis

Total RNA was extracted by modified CTAB method [12]. First strand cDNA was synthesized from 1 μg of total RNA using the PrimerScript® RT reagent Kit with gDNA Eraser (Takara, Japan). The cDNA was diluted tenfold and 1 μl of the diluted cDNA was used as the template for real-time quantitative PCR (Q-PCR) analysis. Primer pairs were designed using primer premier 5 based on NCBI data and transcriptome sequences (Table 1) of mango fruits (Wu et al., 2014). The Q-PCR mixture (20.0 μl total volumes) contained 10 μl of 2x master mix (Thermo Scientific), 0.8 μl of each primer (10 μM), 1 μl of cDNA, and 7.4 μl of RNasefree water. The reactions were performed on a LightCycler 480 instrument (Roche, Diagnostics) with DyNAmo™ ColorFlash SYBR Green qPCR Kit (Thermo Scientific). The q-PCR program was as follows: one cycle of 7 min at 95 °C, followed by 40 cycles at 95 °C for 10 seconds and 60 °C for 20 seconds. Template-less controls for each primer pair were included in each run. The specificity of q-PCR primers was confirmed by melting curve and sequencing of q-PCR products. Relative fold changes were calculated with $2^{-\Delta\Delta CT}$ methods and normalized to that of the actin gene ($Mi\text{Actin}$, HQ585999).

#### Table 1. Primers for real-time PCR analysis

| Gene | NCBI ID | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) | product (bp) |
|------|---------|---------------------------|---------------------------|--------------|
| MiACTIN1 | HQ585999.1 | CCACTGCTGAAAGGGGAAAT | GTGATGGGCTGAAAGGGAC | 192 |
| MiPAL | KF929403.1 | GTTAAGGCGCATTTGAGG | ATCCGTCCCTTTGTCAT | 189 |
| Mi4CL | KF929404.1 | TGGTCCCATCCAGTTCGCC | TCTGGCTAAATCCGGTCA | 86 |
| Mi4CL2 | KF929405.1 | GGCAATATACAGTGAAAGGTG | CTGGGATGGCTGAGAAGC | 160 |
| Mi4CHS1 | KF929407.1 | CCTTCGTGAAGTGGGTTCAT | TAACTTTCTCTTTGAGCCCTAAT | 202 |
| Mi4CHS2 | KF929408.1 | CGGAATCAGGGGAGAGTCTG | TCATTTGTCGGACAGGCCAC | 129 |
| Mi4F3H1 | KF929409.1 | CCACGACACAGGAAAGTATAC | CTGCCGAGGCTATTGACT | 172 |
| Mi4F3H2 | KF929410.1 | TCTACGAACAGTCTCCAT | CTTCGTCGTGTTGCTGCTG | 212 |
| Mi4F3H3 | KF929411.1 | CGGTACCATATCCTCCAG | AACATCGGATCTCCGCTT | 139 |
| Mi4ANS | KF929413.1 | ATGGCAGTGTTATCTACCCG | TGTTGAGGCGGATTGATG | 120 |
| Mi4DFR | KF929412.1 | GCTTACGGATTTGGGATTAA | CTACATCGATTGCGATT | 121 |
| Mi4CL2 | KY386897 | CCCCTACAATACACTCCCT | ATGCCCAACCAGCTTACAAAC | 164 |
| Mi4CHI | KY386895 | GACACTCCCTGCTTCCCT | TCTACCGAGACTCCTACC | 131 |
| Mi4UGT | KY386896 | GAAAGGAGAGGACCGCGAGTG | CTGGGATTGACGACAAATGGA | 178 |
| Mi4UGT2 | KY386898 | TGTTAGCGATGTTGGAGG | GCCTGTATGCGAAACC | 180 |

#### 2.5. Statistical analysis

Statistical analyses were performed using DPS v3.0 software (Hangzhou, China). Duncan multiple range test was used to determine significance of color parameter differences at the 5% level.

### 3. Results

#### 3.1. Pericarp color

Pericarp color of the 14 cultivars was tested, which is expressed as the Hunter L, C and hue angle (H) are shown in Table 2. Significant differences in color parameters were displayed among different cultivars. Basically, the L value of ‘Renong No.1’, ‘Renong No.2’ and ‘Tainong No.1’ is significantly higher than that of other red cultivars and all the greenyellow cultivars. The Chrome value of ‘Renong No.2’, ‘Tainong No.1’ and ‘Renong No.1’ is significantly higher than that of ‘Dashehari’, ‘Guire
No.82’, ‘Guixiang’, ‘Lingshui’ and ‘Qingpi’ mango of greenyellow cultivars. The h value of red cultivars such as ‘Renong No.1’, ‘Renong No.2’, ‘Tainong No.1’, ‘Hongwacheng’ and ‘Zillate’ are significantly lower than that of greenyellow cultivars ‘chinhuang’, ‘lishui’ and ‘qingpi’, while there is no significant difference with ‘R2E2’, ‘Baixiangya’, ‘Guixiang’ and ‘Yuexi No.1’. The lower the hue angle, the redder the fruit skin.

Color index for red grapes (CIRG) [13] is expressed as CIRG = (180-H) / (L+C). The CIRG value of ‘Renong No.1’, ‘Renong No.2’, ‘Hongwacheng’ and ‘Zillate’ were significantly higher than those of the green-yellow cultivars. The CIRG value indicates the degree of skin red coloration, high values mean dark coloration, which is used to evaluate the grape and bayberry coloration [14]. This result was consistent with the visual fruit color phenotypes.

Table 2. The coloration of 14 mango cultivars

| Cultivars     | Light | Chroma | Hue angle | CIRG |
|---------------|-------|--------|-----------|------|
| Tainong No.1  | 83.98 | a      | 2.56      | a    | 86.41 | cd | 1.08 |
| Chinhuang     | 71.25 | de     | 1.71      | bc   | 195.43 | abc | -0.21 |
| Dashehari      | 65.69 | f      | 1.04      | def  | 218.20 | ab | -0.57 |
| White Ivovy    | 77.09 | b      | 1.86      | bc   | 87.39  | cd  | 1.17 |
| Guire No.82   | 72.82 | cd     | 0.98      | f    | 157.85 | abc | 0.30 |
| Guixiang      | 73.38 | bcd    | 0.93      | ef   | 146.31 | abcd | 0.45 |
| Lingshui      | 68.89 | e      | 1.47      | cde  | 232.17 | a   | -0.74 |
| Qingpi        | 74.62 | bc     | 0.88      | f    | 211.19 | ab  | -0.41 |
| Yuexi No.1    | 77.34 | b      | 1.48      | cde  | 92.72  | bcd | 1.11 |
| Renong No.2   | 82.01 | a      | 2.58      | a    | 62.88  | d   | 1.38 |
| Renong No.1   | 84.57 | a      | 2.31      | ab   | 67.37  | d   | 1.30 |
| Hongwacheng   | 76.05 | bc     | 1.64      | bcd  | 77.01  | d   | 1.33 |
| Zillate       | 76.17 | b      | 1.61      | cd   | 64.34  | d   | 1.49 |
| R2E2          | 76.65 | b      | 1.29      | cdef | 119.52 | bcd | 0.78 |

For each cultivar, means within a column followed by different letters are significantly different at p<0.05. Results of ANOVA test (n=10) are presented in table 2.

3.2. Concentration of anthocyanins, chlorophylls, carotenoids, flavonoid and their correlations with pericarp color

Different mango cultivars have certain chlorophyll, carotenoids and flavonoids. Anthocyanins in the 14 cultivars ranged from none to 15.96mg/100g FW, ‘Chinhuang’, ‘Baixiangya’ ‘Guire No.82’ and ‘Guixiang’ contained extremely low or non-detectable levels of anthocyanins, while ‘R2E2’ ‘Renong No.2’ and ‘Lingshui’ accumulated certain anthocyanins (Figure 2).
Contrarily, the contents of chlorophylls in the pericarp of ‘Guire No.82’, ‘Lingshui’ and ‘Qingpi’ were much higher than those in the other cultivars, and the red cultivar ‘Zillate’ also had higher chlorophylls. The content of carotenoids in mango pericarp ranged from 0.049mg/g FW in cultivar ‘Baixiangya’ to 0.17mg/g FW in cultivar ‘R2E2’, these cultivars accumulated higher carotenoids except for baixiangya, chinhuang and qingpi mango. The concentrations of flavonoids in the pericarp of ‘Guire No.82’, ‘Lingshui’, ‘Qingpi’, ‘Guixiang’, and red cultivar ‘Zillate’ were much higher than those in the rest cultivars.

According to the color appearance and content of anthocyanins and chlorophyll, 14 cultivars could be divided into three types: Green-yellow ones that accumulate extremely high chlorophyll and accumulate no or extremely low anthocyanin, including ‘Baixiangya’, ‘Guire No.82’ and ‘Guixiang’, ‘Dashehari’, ‘Lingshui’, ‘Qingpi’. Medium ones, that usually showed yellow, but accumulated certain anthocyanin exposed to sufficient sunlight, including ‘Tainong No.1’ and ‘Chinhuang’. Red ones, that accumulate amount of anthocyanins with decreased chlorophylls, including ‘Renong No.1’, ‘Renong No.2’, ‘R2E2’, ‘Hongwacheng’ and ‘Zillate’.

3.3. Expression of twelve structural genes involved in anthocyanin biosynthesis pathway in different mango cultivars.

To elucidate the molecular mechanisms of red coloration, the transcripts of structural genes correlated anthocyanin synthesis were determined in different mango cultivars at ripe fruits (Figure 3). Basically, for early structure genes, in red and some non-red cultivars like Guixiang, Qingpi and Yuexi No.1 which had higher anthocyanin concentration, the expression of PAL was much higher than in other non-red cultivars and medium cultivars, while the expression of F3’H in red cultivars is much lower than that in green-yellow cultivars. The expression of C4H, 4CL2, ANS, UFGT2 in red cultivars is much higher than that in green-yellow cultivars, while the expressions of CHI, 4CL, CHS1, CHS2, F3H1,DFR, UFGT1 did not showed distinct regularity in red and green-yellow mango cultivars. the exception is the expression of PAL, CHI, CHS2, UFGT1, F3’H in green-yellow cultivar Guixiang and the expression of C4H, 4CL, 4CL2, F3H2 and UFGT2 in ‘Lingshui’ mango is very higher than other green-yellow cultivars.
Figure 3. Expression analysis of anthocyanin biosynthetic genes in the pericarp of 14 mango cultivars. *MiActin* gene was used to normalize expression of the genes under identical conditions. The vertical bars represent standard error of three replicates.

4. Discussion

The expression of F3H, DFR, ANS and UFGT, in the pericarp of red litchi cultivars was much higher than that of non-red cultivars, which indicated that these genes co-ordinately express regulate coloration in litchi fruits. The expression of DFR and UFGT was positively correlated with anthocyanin content in the pericarp [6]. UFGT was found the key gene that made coloration difference between white type and its red sport in grape [15]. The activities of CHI and UFGT in ‘Splendour’ apple were correlated with anthocyanin accumulation during fruit ripening. When fruits were exposed to sunlight, CHS activity was not correlated with anthocyanin accumulation, whereas UFGT was positively correlated with anthocyanin accumulation. The content of anthocyanins was correlated with DFR activity in ‘Delicious’ apple. Hence, the different results might be related to the different genetic background to different research material. In this study, Anthocyanins biosynthetic genes were selected based on the NCBI and sequencing results of transcriptome in ‘Zill’ mango fruits, and gene expression of different varieties were analysed. The expression levels of *MiC4H*, *Mi4CL2*, *MiANS* and *MiUFGT2* in red cultivars were significantly higher than that in green-yellow cultivars. The content of anthocyanin was positively correlated with the expression of PAL, which indicate that PAL maybe key gene affecting fruits coloration in mango. The expression of *MiPAL*, *MiCHI*, *MiCHS2*, *MiF3’H* and *MiUFGT* was higher in non-red Guixiang mango and the expression of *MiC4H*, *Mi4CL*, *Mi4CL2*, *MiF3H2* and *MiUFGT2* was higher in non-red Lingshui mango, which maybe the reasons of these two cultivars had higher content of chlorophyll and covered the red coloration in non-red cultivars.

In different apple cultivar, DFR activity in red cultivars is significantly higher than that in non-red cultivars [16-17]. In this study, expression level of DFR in non-red varieties and Zillate, a red-skinned variety with higher content of chlorophyll content was higher than that in red varieties, this is mainly because fruit coloration in mango is different from that in apple. Anthocyanin synthase (ANS), which catalyzes the conversion of colorless leucoanthocyanins into colored anthocyanins, is a critical enzyme in the anthocyanin biosynthetic pathway [18], the first color compounds of anthocyanins synthesis, plays an important role in the formation of fruit coloration [19]. Kondo et al. reported that synthesis of anthocyanins in the skin of ‘Mutsu’ is related to the expression of ANS and UFGT [20]. In this study, the transcript level of *MiANS* in red mango cultivars is higher than that in non-red cultivars. The
expression of MiUFGT2 gene was significantly positive correlation with the content of anthocyanin, this result is similar to apple's results [20].

In this study, mango varieties in the content of anthocyanins, chlorophyll and fruit coloration had significant difference in different mango cultivars. Concentration of anthocyanins in red cultivars is significantly higher than that in non-red cultivars, there is the exception, non-red cultivar ‘lingshui’ and Yuexi No.1 had certain anthocyanins, to some extent, non-red cultivars had higher chlorophyll content covers the red skin. The red one variety Zillate also contains higher chlorophyll content.

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