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Expression of genes involved in the anthocyanin biosynthesis pathway in white and red fruits of *Fragaria pentaphylla* and genetic variation in the dihydroflavonol-4-reductase gene

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ABSTRACT

Structural and regulatory genes control fruit colors in plants. Real-time quantitative PCR results showed significantly higher expression levels of structural genes (*FpCHS*, *FpDFR*, *FpANS*, and *FpUFGT*) as well as of the regulatory gene *MYB10* in red fruits of *Fragaria pentaphylla* compared to white fruits. These genes were strongly associated with anthocyanin accumulation within fruits. The full-length sequence of the *FpDFR* gene in red fruits of *F. pentaphylla* had a length of 2080 bp, was separated by five introns, and shared 95% homology with the *F. vesca DFR* sequence. Twenty-seven SNPs were detected in the *FpDFR* gDNA sequences between red and white fruits. Among these, transition substitutions were more frequent than transversions (66.7% vs. 33.3%), and a larger number of nucleotide variants existed in introns compared to exons (70.4% vs. 29.6%). A Chi-square test showed only three SNPs significantly associated with fruit color. Combined with structural analyses of the *Fp*DFR protein and an expression analysis of the anthocyanin pathway genes, these results indicate that *trans*-regulation might contribute to color control in *F. pentaphylla*.

1. Introduction

Fruit color results from the accumulation of anthocyanin and is an important quality trait, influencing human consumption (Goto and Kondo, 1991). Recently, anthocyanins have attracted increasing interest due to their important physiological functions, such as antioxidative (Wang et al., 1999; Kähkönen and Heinonen, 2003), antimutagenic (Gasiorowski et al., 1997), and anticancer activities (Koide et al., 1996).

The biosynthetic pathway of anthocyanins has been well-described in various plants, including *Zea mays* L., *Antirrhinum majus* L., *Petunia hybrida* Vilm., and *Arabidopsis thaliana* (L.) Heynh. (Winkel-Shirley, 2001; Tanaka et al., 2008; Dixon and Steele, 1999; Holton and Cornish, 1995). Generally, the following six enzymes are involved in the biosynthesis pathway of anthocyanin: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose-flavonoid 3-*O*-glucosyltransferase (UFGT) (Ma et al., 2009). In addition to structural genes, regulatory genes have been reported to play a vital role in the biosynthesis of anthocyanins in strawberry plants (Streisfeld and Rausher, 2010). Mutations in structural genes and transcription factor genes can affect the biosynthesis of anthocyanin (Streisfeld and Rausher, 2010).

Among structural genes, DFR encodes the first enzyme for anthocyanin biosynthesis in the flavonoid pathway (Griesbach, 2005). DFR controls the direction of carbon flux and catalyzes the conversion of dihydroflavones to unstable leucoanthocyanidins, which are common precursors for the anthocyanin and proanthocyanidin biosynthesis (Wang et al., 2013). The regulation of *DFR* expression has been well documented to change the flower color of Japanese parsley, lotus, and pansy (Hasegawa et al., 2001; Buathong et al., 2013; Li et al., 2014). Moreover, deactivation of DFR has been reported to result in loss of anthocyanins and proanthocyanidin in barley and *Arabidopsis* mutants (Olsen et al., 1993; Shirley et al., 1995).

Fragaria pentaphylla Losinsk is a wild diploid species of the *Fragaria* genus that belongs to the Rosaceae family (Yu, 1974). The

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two different fruit colors (white and red) can be found in *F. pentaphylla* varieties in the field (Yu, 1974). The genome of *F. pentaphylla* is small and is considered a good model for studies of the mechanisms that underlie the production of fruit color. In the present study, we compared the expression of six structural genes and one regulatory gene, which are involved in the biosynthesis pathway of anthocyanin in white and red fruits of *F. pentaphylla*. To determine whether *FpDFR* is involved in the anthocyanin biosynthesis in *F. pentaphylla*, we examined the relationship between the *FpDFR* gene and the contents of both anthocyanin and flavonoids, isolated the cDNA and full-length sequences of the *FpDFR* gene, and analyzed the associations between single nucleotide polymorphism (SNPs) and fruit color. The results of this study provide a basic reference for the regulation of fruit color in strawberry.

2. Materials and methods

2.1. Plant materials and samples collection

Nine individual plants with non-matured white fruits and nine individual plants with red fruits of the diploid *F. pentaphylla* were collected respectively in July 2012 in Mao County, Chengdu City, Sichuan Province, China. The plants were transplanted and grown in a walk-in growth chamber (Taizhou University, Zhejiang Province, China), at day/night temperature of 20 °C/15 °C for 10 h/14 h. When the fruits were matured, two fruits and one young leaf were collected from every plant. The tissues were immediately frozen by liquid nitrogen and stored at −80 °C until analysis.

2.2. Quantitative RT-PCR

Real-time quantitative PCR (qPCR) was performed on the cDNA libraries of three red fruit and three white fruit respectively using specific primers (*CHS*: FP5′ CCGACTACTACT TTCGT ATCACCA 3′, RP5′ ACTACCACCATGTCTTGTCTTGC 3′; *CHI*: FP5′ TGACAATGATACTACCGCTGAC 3′, RP5′ CTGGTGGGAAGGTCTGATCTTT3′; *F3H*: FP5′ TGGAGAGATGTGACAAAGCAGT 3′, RP5′ TCAAATGCCTCCTTCTCTAAACC3′; *DFR*: FP 5′ CCAAGGACCCTGAGAATGAA 3′, RP 5′ TTACTCTCCGGCAAAATTCG 3′; *ANS*: FP5′ TGTGGCAACAAGTGAGTATGC3′, RP5′ ACCGACCTCCTTCTCCAGCCT3′; *UFGT*: FP5′ AATGGCATGCTTAAGAGTTTGGA3′, RP5′ CTGTTGTGCGAGTTGTTTTAGTG 3′; *MYB10*: FP5′ AGATGCAGGAAGAGCTGTAGA C 3′, RP5′ TTCCTAGAAGCTTATGAAGCCT 3′) with the SYBR Green Master Mix (SYBR® Premix Ex Taq™, TaKaRa, Co. Ltd., Dalian, China) on an Illumina Eco Real-Time PCR System (Illumina Inc., San Diego, CA, USA). Reactions were performed in triplicate using 12.5 μL of 10 × buffer, 0.4 mol L^{-1} of each primer, 2 μL of diluted cDNA, and nuclease-free water to a total volume of 20 μL. The real-time qPCR assay conditions were as follows: pre-incubation at 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and final extension at 72 °C for 30 s. The length of the PCR product was not less than 100 bp. Fluorescence was measured at the end of each annealing step. Results from the real-time qPCR assays were normalized using the Ct value corresponding to the reference gene (18S rRNA) using specific primers (FP 5′ ACCGTTGATTCGCACAATTGGTCATCG 3′ and RP 5′ TACTGCGGGTGGCAATCGGACG 3′). Relative expression levels were quantified using the $2^{-\triangle\triangle}$ method (Pfaffl, 2001).

2.3. Determination of total anthocyanin content

Three red fruits and three white fruits, which were collected from the same plants used for cDNA cloning, were separately ground and extracted in 15 mL of methanol acidified with 1.0% HCl at room temperature for 24 h. The extract was centrifuged at 12000×*g* for 5 min, and the supernatant was transferred to a new centrifuge tube. The total anthocyanin concentration was determined using a modified pH differential method described previously (Garzon and Wrolstad, 2002). A general spectrophotometer (T6 New Century; Purkinje General Instrument Co. Ltd., Beijing, China) was used to measure the absorbance of the extract solution in buffers of pH 1.0 and pH 4.5 at 520 nm and 700 nm, respectively. Total anthocyanins content was expressed as mg of cyanidin-3-glucoside per g of dry weight of fruit. Total anthocyanins = A × MW × B × 100 × V/ξ, where $A = [(A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}]$, MW indicates molecular weight 433.2 g mol^{-1}, B indicates the dilution ratio, V indicates the final volume, and ξ indicates the molar extinction coefficient of 22,400. All samples were analyzed in triplicate.

2.4. Determination of total flavonoid content

The total flavonoid content was determined using an aluminum chloride colorimetric assay (Jia et al., 1999). A specific volume of extract was placed in a 10-mL volumetric flask and diluted with distilled water to 5 mL. Then, 0.3 mL of $NaNO_2$ (1:20) and 3 mL of $AlCl_3$ (1:10) were added after 5 min, and after a further 6 min, 2 mL of 1 M NaOH were added. Then, distilled water was added to obtain a total volume of 10 mL. The solution was mixed again, and a general spectrophotometer (T6 New Century, Purkinje General Instrument Co., Beijing, China) was used to measure absorbance at 510 nm. Total flavonoid content was expressed as mg rutin equivalents per g of fresh mass. All samples were analyzed in triplicate.

2.5. Cloning and characteristics of FpDFR cDNA

Total RNA was extracted from red fruits, using the RNeasy Plant Mini Kit (Qiagen Co. Ltd., Hilden, Germany). The RNA concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and the absorbance ratios at 260 nm and 280 nm were calculated to evaluate the quality of RNA and to diluted RNA to 1 μg μl^{-1}. A cDNA library was synthesized using the PrimeScript™ Double Strand cDNA Synthesis Kit (Takara Co. Ltd., Dalian, China) with oligo dT primers.

The full-length coding sequence for the DFR protein was PCR amplified. For this, a cDNA library was used, which was amplified using total RNA extracted from a red fruit as template. PCR amplification was conducted with specific primers (FP 5′ ATGGGATCGGAGTCCGAATCC 3′ and RP′ TTAGCCAGTGACTTCGACATGG 3′), designed according to the potential cDNA region of the genomic DNA of *F. pentaphylla*, using the following PCR amplification procedure: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min), and a final extension at 72 °C for 10 min. PCR products were analyzed with 1.0% agarose gels, single fragments were recovered from gels, and purified using a DNA purification kit (BioTeke Co. Ltd., Beijing, China). The purified gene product was ligated into the pMD18-T vector (Takara Co. Ltd., Dalian, China) and transformed into *E. coli* DH 5α for subsequent sequencing. PCR products were analyzed on 1.0% agarose gels and recovered from the gel via DNA purification kit (BioTeke Co. Ltd., Beijing, China). PCR products were directly sequenced by Biosune Co. Ltd. (Shanghai, China) via the ABI 373X (ABI, Carlsbad, CA, USA). The nucleotide sequence of the *FpDFR* cDNA was analyzed via BLASTN search against the GenBank database of the National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov/BLAST/.

Both the theoretical isoelectric point and molecular weight were calculated online (http://www.expasy.ch/tools/pi_tool.html). The