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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 FpDFR 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 *fl*avonoid 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 $\mu l^{-1}$. 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

ORF of the full-length DFR was identified via Genscan (http://genes.mit.edu/GENSCAN.html) and motifs were found with Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The secondary structure of the predicted DFR protein was constructed with the PredictProtein tool (http://www.predictprotein.org/) and the three-dimensional structure of the predicted DFR was modeled via SWISS-MODEL with Automated model (http://swissmodel.expasy.org/). A phylogenetic analysis of the FpDFR protein was performed based on the deduced protein sequence using the NJ method, which was implemented in MEGA 5.0.

### 2.6. Cloning of FpDFR genomic DNA and SNP analysis

Young leaves from F. pentaphylla with red fruits were collected and genomic DNA was extracted, following the protocol of the utilized DNA extraction kit (Dingguo Co. Ltd., Beijing, China). PCR amplification was conducted with specific primers (FP 5′ GATCGTATCAGACATTCTCC 3′ and RP 5′AATGATGAATGAGACGTCCTA 3′), which were designed in accordance with the DFR gene sequence of F. vesca (GenBank accession No. NM_001305268.1). The following PCR conditions were utilized: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min), followed by a final extension at 72 °C for 10 min. Fragments were amplified using the Phusion® High-Fidelity DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA). PCR products were analyzed with 1.0% agarose gels and recovered from the gel via DNA purification kit (BioTeke Corporation, Beijing, China). PCR products were directly sequenced by Biosune Co. Ltd. (Shanghai, China) via the ABI 373X (ABI, Carlsbad, USA). The sequence was then mapped to determine the positions of SNPs. Significance levels of associations between SNPs and color trait were evaluated via SPSS software (version 19.0).

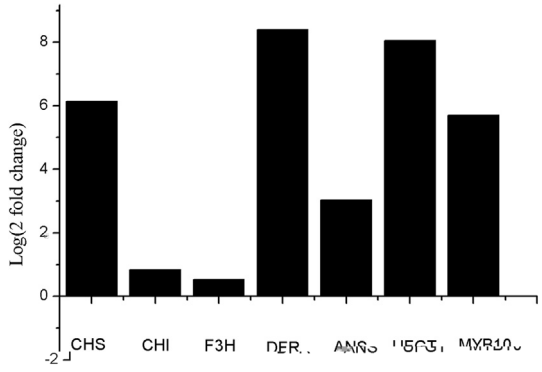


**Fig. 1.** $Log_2$-fold-change of anthocyanin biosynthesis pathway genes expression. The results represent the relative expression in red fruits vs. white fruits of Fragaria pentaphylla. CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; UFGT: UDP-glucose-flavonoid 3-O-glucosyltransferase.

## 3. Results

### 3.1. Expression of genes involved in the anthocyanin biosynthesis pathway and their correlation with total anthocyanin and flavonoid contents in red and white fruits

Both the expression levels of structural genes (FpCHS, FpDFR, FpANS, and FpUFGT) and the regulatory gene MYB10, which is involved in the pathway of anthocyanin biosynthesis in red fruits were significantly higher than those in white fruits, while the expression of the remaining structural genes (FpCHI and FpF3H) were similar between red fruits and white fruits (Fig. 1).

The total anthocyanin content of red fruits was 12.36 ± 0.12 mg/100 g FW, while that of white fruits was almost undetectable (0.06 ± 0.08 mg/100 g FW) (Fig. 2a) and was approximately 200-fold lower than that of red fruits of F. pentaphylla. However, the total flavonoid content of red F. pentaphylla fruits was similar to that of white fruits (Fig. 2b).

### 3.2. Correlation between SNPs of FpDFR and fruit color

The full length of the FpDFR gene sequence of red F. pentaphylla fruits was 2080 bp. This sequence was submitted to GenBank under the accession number KR075887.1. A comparison of the full-length genomic sequence of FpDFR with that of the cDNA in the red F. pentaphylla fruits revealed six exons of 118, 170, 195, 160, 193, and 217 bp, separated by five introns of 91, 495, 108, 84, and 79 bp, respectively. It shared 95% homology with the DFR gene of F. vesca (GenBank accession number AY017480).

Twenty-seven SNPs were detected in FpDFR gDNA sequences between red and white fruits of F. pentaphylla (Table 1). Among these, transition substitutions were more frequent than transversions (66.7% vs. 33.3%) and more nucleotide variants were located in introns than in exons (70.4% vs. 29.6%) (Table 1). Transition between G and A was more common than between C and T. For transversions, the substitutions were as follows (arranged in order of decreasing frequency): C and G, T and G, and C and A. Transversions between C and A were not detected. A Chi-square

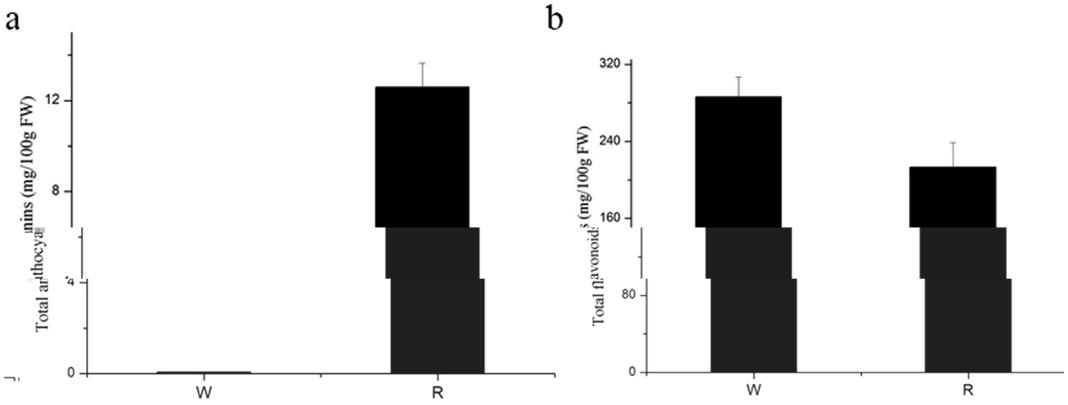


**Fig. 2.** The total anthocyanin content (a) and total flavonoid content (b) in red (R) and white (W) fruits of Fragaria pentaphylla. Error bars indicate standard deviations of three independent biological samples.

**Table 1**
SNPs in DFR gene between red and white fruits and their association with fruit color in Fragaria pentaphylla.

| Position | Location | Allel | Frequency | | Chi-square (p value) | Type of single-base substitution |
|---|---|---|---|---|---|---|
| | | | Red fruit | White fruit | | |
| 43 | Exon | C | 0.444 | 0.889 | 2.492 (0.114) | Transversion |
| | | G | 0.556 | 0.111 | | |
| 161 | Intron | C | 0.444 | 0.111 | 2.492 (0.114) | Transversion |
| | | G | 0.556 | 0.889 | | |
| 248 | Exon | A | 0.556 | 1.000 | 6.923 (0.009) | Transition |
| | | G | 0.444 | 0.000 | | |
| 362 | Exon | C | 0.222 | 0.222 | 0.000 (1.000) | Transversion |
| | | G | 0.778 | 0.778 | | |
| 468 | Intron | A | 1.000 | 0.556 | 5.143(0.023) | Transition |
| | | G | 0.000 | 0.444 | | |
| 471 | Intron | A | 0.000 | 0.222 | 2.250 (0.134) | Transition |
| | | G | 1.000 | 0.778 | | |
| 596 | Intron | C | 0.000 | 0.222 | 2.250 (0.134) | Transversion |
| | | G | 1.000 | 0.778 | | |
| 686 | Intron | A | 0.778 | 1.000 | 2.250 (0.134) | Transition |
| | | G | 0.222 | 0.000 | | |
| 694 | Intron | C | 0.667 | 0.889 | 1.286 (0.257) | Transition |
| | | T | 0.333 | 0.111 | | |
| 712 | Intron | C | 1.000 | 0.778 | 2.250 (0.134) | Transition |
| | | T | 0.000 | 0.222 | | |
| 715 | Intron | C | 1.000 | 0.778 | 2.250 (0.134) | Transversion |
| | | G | 0.000 | 0.222 | | |
| 736 | Intron | A | 1.000 | 0.778 | 2.250 (0.134) | Transition |
| | | G | 0.000 | 0.222 | | |
| 775 | Intron | C | 0.000 | 0.222 | 2.250 (0.134) | Transversion |
| | | G | 1.000 | 0.778 | | |
| 794 | Intron | G | 1.000 | 0.778 | 2.250 (0.134) | Transversion |
| | | T | 0.000 | 0.222 | | |
| 808 | Intron | G | 0.000 | 0.222 | 2.250 (0.134) | Transversion |
| | | T | 1.000 | 0.778 | | |
| 830 | Intron | A | 0.000 | 0.333 | 3.60 (0.058) | Transversion |
| | | T | 1.000 | 0.667 | | |
| 1029 | Intron | C | 0.778 | 1.000 | 2.250 (0.134) | Transition |
| | | T | 0.222 | 0.000 | | |
| 1340 | Intron | A | 0.222 | 0.000 | 2.250 (0.134) | Transition |
| | | G | 0.778 | 1.000 | | |
| 1349 | Intron | C | 1.000 | 0.778 | 2.250 (0.134) | Transition |
| | | T | 0.000 | 0.222 | | |
| 1381 | Intron | C | 0.778 | 1.000 | 2.250 (0.134) | Transition |
| | | T | 0.222 | 0.000 | | |
| 1402 | Exon | A | 0.000 | 0.444 | 5.143 (0.023) | Transition |
| | | G | 1.000 | 0.556 | | |
| 1450 | Exon | C | 1.000 | 0.778 | 2.250 (0.134) | Transition |
| | | T | 0.000 | 0.222 | | |
| 1621 | Intron | A | 0.000 | 0.222 | 2.250 (0.134) | Transition |
| | | G | 1.000 | 0.778 | | |
| 1656 | Exon | A | 0.222 | 0.000 | 2.250 (0.134) | Transition |
| | | G | 0.778 | 1.000 | | |
| 1668 | Exon | A | 0.222 | 0.222 | 0.000 (1.000) | Transition |
| | | G | 0.778 | 0.778 | | |
| 1703 | Exon | A | 0.000 | 0.333 | 3.60 (0.058) | Transition |
| | | G | 1.000 | 0.667 | | |
| 1908 | Intron | C | 0.778 | 1.000 | 2.250 (0.134) | Transition |
| | | T | 0.222 | 0.000 | | |

test showed that only three SNPs significantly associated with fruit color (Table 1). The SNP at 248 bp was classified as a nonsense mutation, resulting in an amino acid substitution of lysine for glutamic acid in white fruits of F. pentaphylla, while SNPs at positions 468 and 1402 bp were classified as synonymous mutations.

### 3.3. Characterization of FpDFR cDNA and proteins

The full-length cDNA of FpDFR in red fruits of F. pentaphylla had a length of 1050 bp, encoding a polypeptide of approximately 350 amino acid residues, which corresponds to a molecular mass of 38.992 kDa, with a theoretical pI of 6.2 (Fig. 3a; sequence was submitted to GenBank under accession number KR075887). The nucleotide sequence of FpDFR cDNA in red fruits of F. pentaphylla shared significant homologies with the DFR cDNA of Fragaria vesca L. cultivar Alexandria (KC894050.1, 99% similarity), Fragaria × ananassa Duch. cultivar Elsanta (KC894054.1, 99% similarity), Fragaria × ananassa cultivar Chandler (AF029685.1, 99% similarity), Rosa rugosa Thunb. (KM203111.1, 92% similarity), a Rosa hybrid cultivar (AB490072.1, 91% similarity), and Rosa chinensis Jacquem. (KF734592.1, 90% similarity).

An analysis of the secondary structure of the predicted FpDFR protein revealed that all examined FpDFRs contained an NAD (P)-binding domain. Furthermore, secondary structure analysis of the predicted FpDFR revealed that this predominantly consisted of β-pleated sheets (50.29%), followed by α-helices (36.29%), and random coils (13.43%) (Fig. 3b).

The quality of the predicted three-dimensional structure of the

FpDFR protein in red fruits of F. pentaphylla was revealed via comparison with data in the Protein Data Base Structures database, and the resulting global model quality estimate was 0.83, suggesting high quality of the predicted model (Fig. 3c).

The phylogenetic tree based on the nucleic acid sequences of FpDFR in red fruits of F. pentaphylla and other nucleic acid sequences of DFR of various plant species are shown in Fig. 4. Analysis showed the FpDFR sequence in red fruits of F. pentaphylla to be closely related to the DFR nucleic acid sequence of F. vesca (Fig. 4).

## 4. Discussion

Here, the detected expression levels of four structural genes (FpCHS, FpDFR, FpANS, and FpUFGT) and the regulatory gene FpMYB10 in red fruits of F. pentaphylla were significantly higher

and F. vesca (Shulaev et al., 2011). However, the function of DFR to determine plant fruit color varies among plant species (Wang et al., 2004; Ju et al., 1997). Wang et al. (2013) reported that DFR gene expression has a significant relationship with the anthocyanin content in the purple sweet potato. However, Wang et al. (2004) reported that DFR gene expression has no relationship with the anthocyanin content in the pericarp of Litchi. Ju et al. (1997) found that 'Delicious,' 'Golden delicious', and 'Indo' apples also contained relatively high DFR activity, although they did not accumulate anthocyanins. Salvatierra et al. (2013) reported that DFR gene expression in two botanical forms of Fragaria chiloensis Duch. spp. chiloensis was only equal in red and white botanical forms during the ripening stages. In our study, the strong correlation between the expression of the FpDFR gene and the anthocyanin content in F. pentaphylla indicates FpDFR expression to be a key step in the anthocyanin biosynthesis. Lin et al. (2013) verified via RNAi technology, that DFR is a key gene in the pathway of anthocyanin biosynthesis in F. × ananassa fruits.

SNPs have been revealed as the most common source of genetic variation in eukaryotic species and consequently, have become an important marker for genetic studies, such as identifying candidate genes that affect diverse phenotypes (Wondji et al., 2007). In this study, we found that exon and intron sequences showed high similarity and only 27 SNPs were detected in a genomic DNA FpDFR gene comparison between red and white fruits of F. pentaphylla. Transition substitutions were more common than transversions (with a ratio of transitions to transversions close to 2:1), which was also observed for Drosophila (Moriyama and Powell, 1996), the mosquito Anopheles funestus (Wondji et al., 2007), and humans ( ).

TAong eall-244.7(eNPs) anly ther12.9(ree-341.6(signi)]TJ/F31Tf10.4(7140TD()Tj/F11Tf.61560TD[(cant)l14.2(oy-396.as)socite)9.(d-24

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2017.04.004.

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