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Anthocyanin Accumulation and Related Gene Expression Profile in 'Red Zaosu' Pear and Its Green Mutant

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Abstract: Red-skinned pear is a promising commercial fruit due to its attractive appearance and nutritious value. Anthocyanin is the determinant of the red coloration of the pear peel. However, differences in anthocyanin accumulation exist among red pear cultivars with different genetic backgrounds. In this study, we analyzed the anthocyanin content and gene expression patterns in the fruits and different tissues of the red pear 'Red Zaosu' at different developmental stages and found a difference in anthocyanin accumulation between 'Red Zaosu' pear and its green mutant. The data showed that the expression profiles of transcripts that encoded critical anthocyanin biosynthetic genes were basically consistent with a tendency to a decreased anthocyanin content during fruit development, indicating that a synergistic effect of these genes was responsible for anthocyanin biosynthesis and regulation. Tissue-specific expression analysis of anthocyanin biosynthetic genes showed that they could be expressed in all tissues but at different levels. *PbF3H*, *PbDFR*, and *PbANS* were mainly expressed during the early flowering period, which explained the reduced levels of anthocyanin content in petals. Additionally, the content of anthocyanins and the expression levels of *PbDFR*, *PbANS*, and *PbMYB10* significantly decreased in the green mutant of 'Red Zaosu', suggesting that *PbDFR*, *PbANS*, and *PbMYB10* probably play a decisive role in determining the skin coloration of 'Red Zaosu' and its green mutant.

Keywords: anthocyanin; gene expression; red pear; green mutant; coloration

Citation: Zhang, Y.; Li, S.; Gu, X.; Lei, D.; Zhao, B.; Tang, H.; Lin, Y.; Wang, Y.; Li, M.; Chen, Q.; et al. Anthocyanin Accumulation and Related Gene Expression Profile in 'Red Zaosu' Pear and Its Green Mutant. *Agriculture* **2021**, *11*, 898. https://doi.org/10.3390/agriculture11090898

Academic Editors: Stefano Giovanni La Malfa and Mario Di Guardo

Received: 9 August 2021
Accepted: 17 September 2021
Published: 18 September 2021

1. Introduction

Fruit skin color is one of the key determinants of fruit quality and commercial value. At present, pears on the market have green, yellow, red, and brown skin. The degree of red coloration in the skin of pears is determined by the content and composition of anthocyanins. Cyanidin-3-galactoside is the major anthocyanin responsible for the red appearance [1–3]. Anthocyanins play important roles during plant growth and development and are involved in a wide range of biological processes, such as the attraction of pollinators and seed dispersers and the protection against biotic and abiotic stresses [4,5]. More importantly, increasing evidence reveals that anthocyanins possess health-promoting properties including diabetes alleviation, obesity control, age-related disease prevention, anti-inflammatory and anti-carcinogenic action, all of which are more or less associated with their potent antioxidant activity [6–8]. Thus, pears with a red skin in addition to their good taste and flavor are more attractive to consumers.

Anthocyanins are synthesized via the flavonoid branch of the general phenylpropanoid pathway, which is controlled by structural genes encoding enzymes that catalyze each

step of the biosynthetic pathway (Figure 1) [9]. So far, several structural genes including *PAL* (phenylalanine ammonia-lyase), *CHS* (chalcone synthase), *CHI* (chalcone isomerase), *F3H* (flavanone 3-hydroxylase), *DFR* (dihydroflavonol 4-reductase), *ANS* (anthocyanidin synthase), and *UFGT* (UDP glucose: flavonoid 3-O-glucosyltransferase) have been well characterized in model plants and fruits, such as *Arabidopsis* [10], petunia [11], apple [12], pear [13], and strawberry [14].

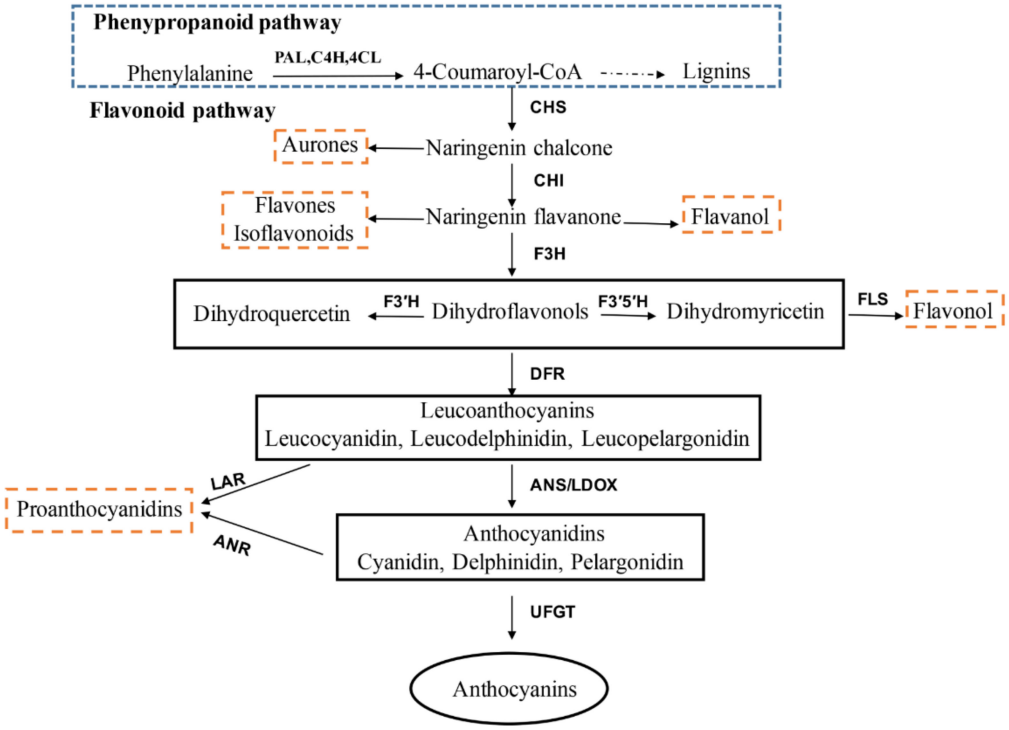

Figure 1. Simplified scheme of the anthocyanin biosynthesis pathway. PAL, phenylalanine ammonia-lyase, C4H, cinnamate 4-hydroxylase, 4CL, 4-coumarate CoA ligase, CHS, chalcone synthase, CHI, chalcone isomerase, F3H, flavanone 3-hydoxylase, DFR, dihydroflavonol 4-reductase, ANS, anthocyanidin synthase, also called leucoanthocyanidin dioxygenase, LDOX, LAR, leucoanthocyanidin reductase, ANR, anthocyanidin reductase. UFGT, flavonoid 3-O-glucosyltransferase.

The spatio-temporal expression of these structural genes is modulated by transcription factors, especially by the MYB-bHLH-WD repeat (MBW) ternary complex consisting of R2R3-MYB, bHLH (basic helix-loop-helix), and WD40 [15]. Of these, R2R3-MYB transcription factor was often found to play a crucial role in anthocyanin accumulation by directly binding to the promoters of structural genes [16]. In pear, PyMYB10 and PyMYB10.1 could activate *DFR* gene expression to positively modulate anthocyanin biosynthesis through interaction with PybHLH [17,18]. In addition, PyMYB114 was identified by linkage to the quantitative trait loci (QTL) for red skin color. PyMYB114 and PyMYB10 have an additive effect that enhances anthocyanin accumulation [19]. This suggest that complicated molecular mechanism exists for fruit coloration.

'Red Zaosu', a bud sport of the green-skinned 'Zaosu' pear, is a hybrid of the Asian pear and *Pyrus communis*. Its phenotype shows red young leaves, flowers, and fruits. In this study, the molecular mechanism of anthocyanin regulation in 'Red Zaosu' were investigated. Additionally, we found that reverse mutations in some branches led to a loss of color in the fruit skin after grafting of 'Red Zaosu' (Figure 2); therefore, mature fruits of 'Red Zaosu' and its green mutant were obtained from the same tree to explore their differential anthocyanin accumulation. These analyses contribute to a better understanding of the red coloration in pear.

Figure 2. Different tissues and organs of 'Red Zaosu'. (**a**) New shoots; (**b**) flowers; (**c**) mature fruits; (**d**) pear tree 'Red Zaosu' bearing red fruits and green mutants within a single tree.

2. Materials and Methods

2.1. Plant Materials

'Red Zaosu' pears were grown in a commercial orchard in Langzhong city, Sichuan province, China. The petals at four different stages of flower development (S1, bud stage; S2, initial flowering stage; S3, semi-open flowering stage; S4, full-bloom stage) were sampled. Besides, the anthers and receptacles at the full-boom stage and young leaves were collected. Fruits were harvested 30, 60, 90, 120 days after full bloom (DAFB). Mature fruits of 'Red Zaosu' and its green mutant were obtained from the same tree. The peels of the fruits were peeled off. All the tissues and peels were instantly frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Extraction and Measurement of Total Anthocyanins

Samples were quickly ground into fine powder in liquid nitrogen. Then, anthocyanins were extracted following previously described procedures [20]. Quantification of the extract was carried out according to the pH differential method by using a UV-visible spectrophotometer at 496 nm and 700 nm [21]. The total anthocyanin content was expressed as μg of cyanidin-3-galactoside per g fresh tissue [1].

2.3. RNA Extraction and cDNA Synthesis

Total RNA was isolated from each sample according to the modified CTAB (cetyltrimethylammonium bromide) method [22]. After gel electrophoresis and spectrophotometry to test the quality and yield of the extracted RNA, 1 μg of high-quality total RNA was reverse-transcribed into the first strand cDNA by using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China).

2.4. Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) primers were designed using Beacon Designer 7 (Table S1). The specificity of primer amplification was confirmed by the melting

curve, fragment size, and sequencing. The reaction mixture (25 µL) comprised 12.5 µL of SYBR Premix Ex Taq™ (Takara, Dalian, China), 2 µL of undiluted cDNA, 1 µL of each primer (10 µM), and RNase-free water. qRT-PCR was performed using 96-well plates on the CFX96 real-time PCR system (Bio-Rad, USA) with the following program: 95 °C for 30 s; 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Template-free controls were included in each run to check potential reagent contamination. The relative transcript levels of genes involved in anthocyanin biosynthesis were determined according to the $2^{-\Delta\Delta Ct}$ algorithm. *PbACTIN* (Genbank ID: CN938023) was used as the reference gene to normalize the raw data [23]. Three biological replications with three technical replications for each sample were used for qRT-PCR analyses.

2.5. Statistical Analysis

All statistical analyses were performed with IBM SPSS Statistics version 23.0 (IBM, New York, NY, USA). Data are presented as means ± standard error. Significant differences were based on one-way ANOVA followed by the Duncan's multiple range test ($p < 0.05$).

3. Results

3.1. Anthocyanin Accumulation during 'Red Zaosu' Fruit Development

Fruits from the cultivar 'Red Zaosu' were collected to investigate the pattern of anthocyanin accumulation at four developmental stages. The results showed that the highest anthocyanin content was 345 µg/g FW at 30 DAFB and then had a tendency to decrease slightly during fruit development, reaching the lowest value of 312 µg/g FW at the mature stage (120 DAFB), which was in accordance with the phenotype change. Clearly, the skin of 'Red Zaosu' fruit was fully red at the early stages of fruit development, while red-green stripes appeared at late stages of fruit development (Figure 3).

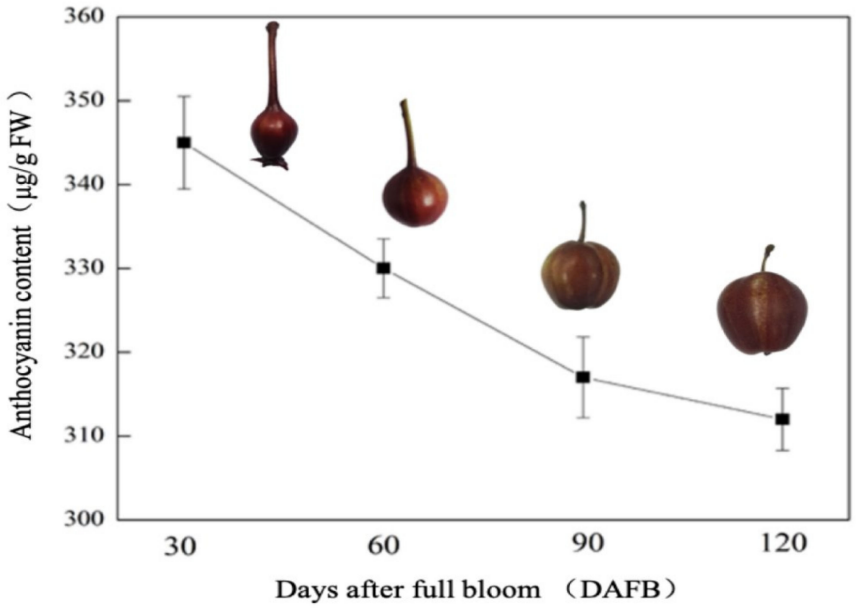

Figure 3. Anthocyanin content of 'Red Zaosu' at different fruit development stages. Fruits were harvested 30, 60, 90 and 120 days after full bloom (DAFB).

3.2. Expression Profile of Anthocyanin Biosynthetic Genes during 'Red Zaosu' Fruit Development

The expression profiles of transcripts that encoded six critical anthocyanin biosynthetic genes (*PbPAL*, *PbCHS*, *PbCHI*, *PbF3H*, *PbDFR*, and *PbANS*) in 'Red Zaosu' during the different fruit developmental stages were detected by qRT-PCR, as shown in Figure 4. The expression of *PbPAL*, *PbCHS*, *PbF3H*, *PbDFR*, and *PbANS* generally showed a rise-drop trend and reached the highest levels at 60 DAFB. However, the expression of CHI peaked at the early stage (30 DAFB) and then dramatically declined and remained low. It was

apparent that the expression tendency of these tested genes was basically consistent with the variation of anthocyanin content. Moreover, the expression of all genes except *CHS* showed no significant difference at the late stages of fruit development (90 and 120 DAFB), suggesting that anthocyanin biosynthesis tended to reach a plateau.

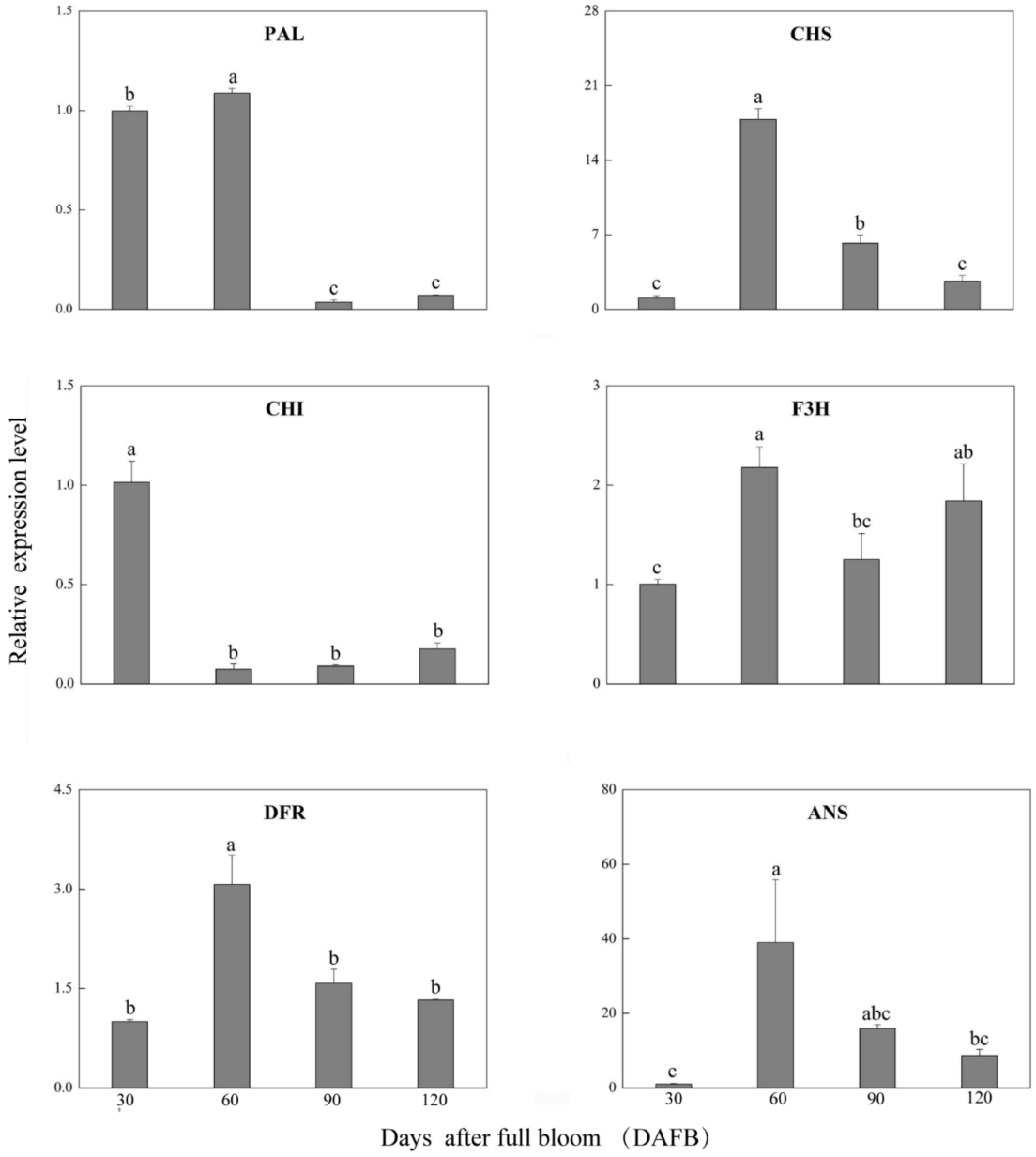

Figure 4. Expression patterns of the anthocyanin biosynthetic genes at different fruit development stages. Different lowercase letters indicate significant differences by ANOVA and Duncan's test at $p < 0.05$.

3.3. Expression Profile of Anthocyanin Biosynthetic Genes in 'Red Zaosu"s Different Tissues

The transcription levels of structural genes in the anthocyanin biosynthetic pathway were determined in different 'Red Zaosu' tissues. Clearly, the transcript levels of *PbDFR* and *PbANS* were remarkably higher in the anthocyanin-rich young leaf than in other tissues. *PbPAL*, *PbCHS*, *PbCHI* were mainly expressed in petals and young leaves, while *PbF3H* showed a significant transcript accumulation in petals and anthers. In addition, the expression of these genes was also detected in petals at four different stages of flower development. *PbDFR* and *PbANS* expression gradually decreased from the bud stage (S1) to the full-bloom stage (S4), which is in accordance with the flower phenotype, while

PbPAL, *PbCHS*, *PbCHI* showed a tendency to first drop and then rapidly increase and finally peaked at the full-bloom stage (S4). *PbF3H* was mainly expressed at the bud stage (S1) and initial-flowering stage (S2) (Figure 5).

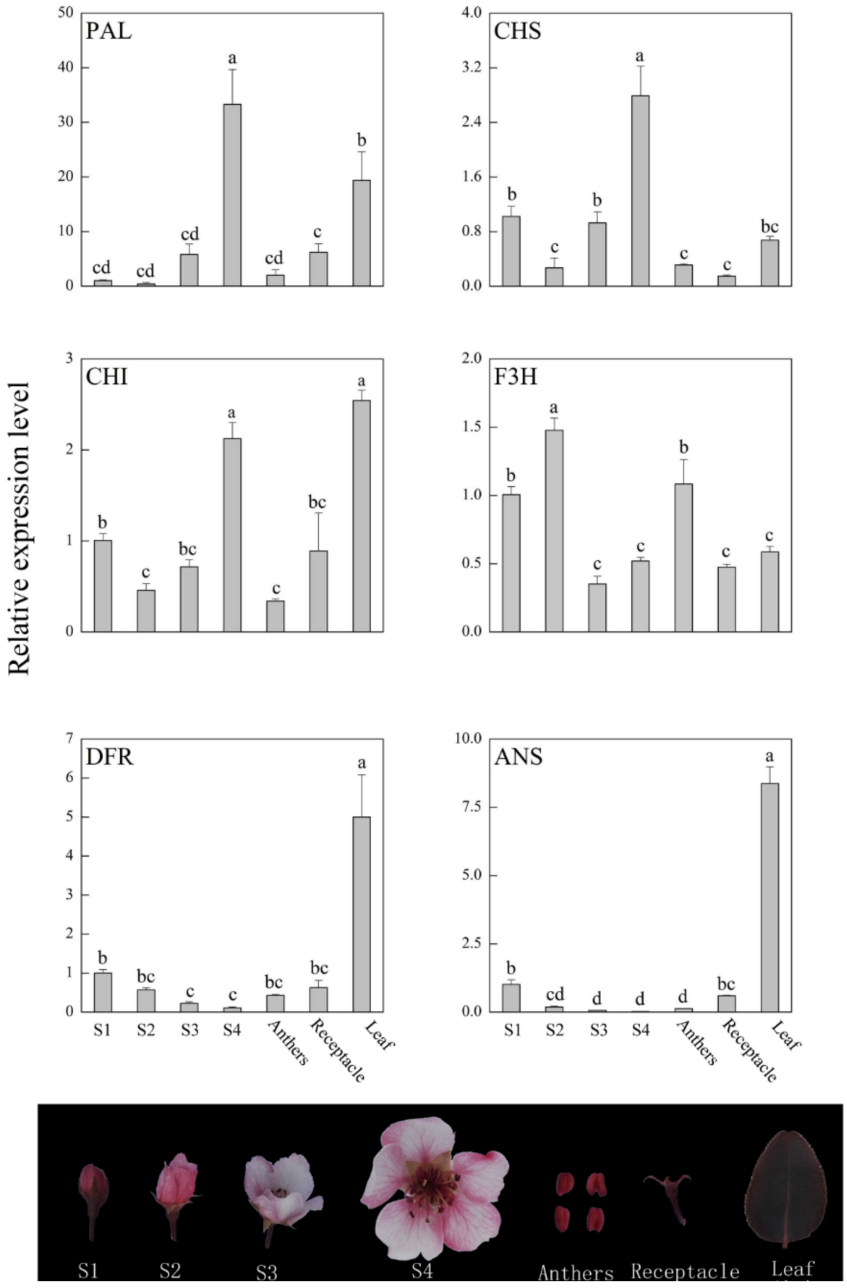

Figure 5. Expression patterns of the anthocyanin biosynthetic genes in different tissues. S1, bud stage; S2, initial flowering stage; S3, semi-open flowering stage; S4, full-bloom stage. Different lowercase letters indicate significant differences by ANOVA and Duncan's test at $p < 0.05$.

3.4. Anthocyanin Content in Ripe Fruits of 'Red Zaosu' and Its Green Mutant

The anthocyanin contents in ripe fruit peels of 'Red Zaosu' and its green mutant from the same tree were measured. Reverse mutation in 'Red Zaosu' caused a severe color loss in the fruit skin, compared to the red-striped skin of 'Red Zaosu' (Figure 6a). As shown in Figure 6b, green mutants only contained 7.8 μg/g of FW anthocyanins, an amount noticeably lower than that in 'Red Zaosu' (87 μg/g FW).

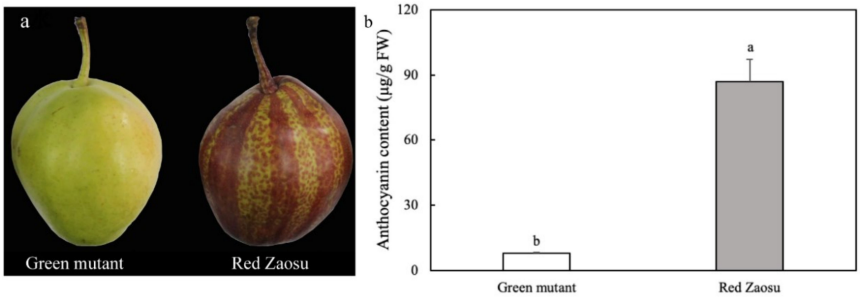

Figure 6. Anthocyanin accumulation in 'Red Zaosu' and its green mutant. (**a**) Fruit skin color of 'Red Zaosu' and its green mutant. (**b**) Anthocyanin content in 'Red Zaosu' and its green mutant.

3.5. Expression of Genes Involved in Anthocyanin Biosynthesis in Ripe Fruits of 'Red Zaosu' and Its Green Mutant

To explore the phenotypic differences between 'Red Zaosu' and its green mutant, the expression pattern of important structural and regulatory genes involved in pear anthocyanin accumulation were analyzed. The RT-qPCR results showed that the expression of the transcriptional factor *PbMYB10* and the structural genes *PbDFR*, *PbANS*, and *PbPAL* remarkably decreased in the green mutant, but that of other three structural genes (*PbCHS*, *PbCHI*, and *PbF3H*) showed no significant difference between the green mutant and 'Red Zaosu' (Figure 7). *PbMYB10* has been widely reported to play a positive role in the regulation of anthocyanin biosynthesis by activating structural genes in red pears. Hence, our results indicated that a reduced expression of *PbMYB10* might decrease the expression of structural gene, eventually leading to color loss in 'Red Zaosu'.

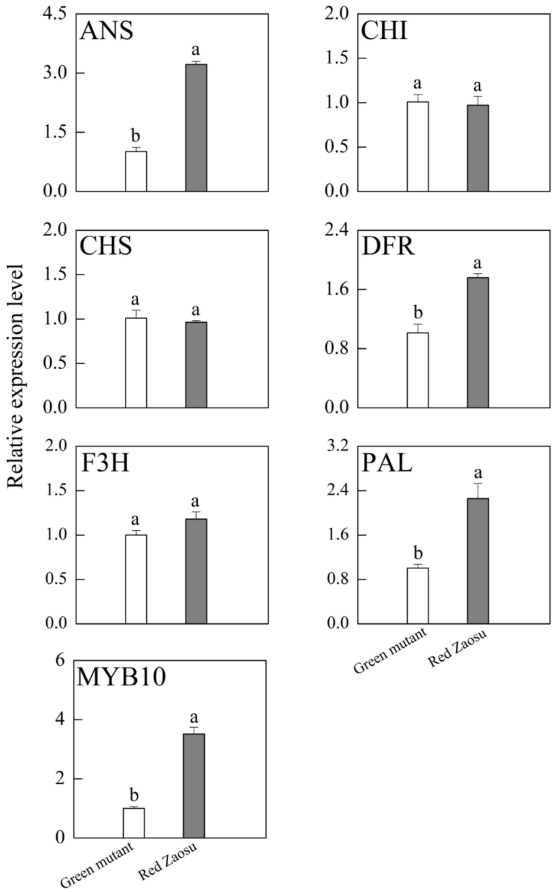

Figure 7. Expression patterns of the anthocyanin biosynthetic genes and *MYB10* in 'Red Zaosu' and its green mutant. Different lowercase letters indicate significant differences by ANOVA and Duncan's test at $p < 0.05$.

4. Discussion

The red-skinned pear cultivars are more appealing to consumers due to their attractive skin color and potential nutritional value. However, it is hard to obtain a red peel color in oriental pear. Mutation selection is one of the most efficient approaches to obtain red-skinned pear cultivars, like the 'Red Zaosu' pear, originated from a red mutant of 'Zaosu' [3]. The concentration and the composition of anthocyanin are the determinants of fruit red coloration, which is controlled by structural genes and regulatory genes in the anthocyanin biosynthetic pathway [24,25]. Here, anthocyanin accumulation and the expression profile of anthocyanin biosynthetic genes in 'Red Zaosu' were systematically analyzed. The results showed that 'Red Zaosu' fruit presented the peak value of anthocyanin contents at the early developmental stages; anthocyanin levels then decreased at maturity (Figure 3), similar to other red occidental pears, such as 'Starkrimson', 'Palacer', 'Red Sichou', and 'Red Bartlett' [24,25]. Hence, for these cultivars, the fruit red skin color partly faded towards harvest. This may be due to a combination of decreased anthocyanin biosynthesis, environmental changes, and natural turnover [26].

It has been documented that the expression of structural and regulatory genes for anthocyanin biosynthesis are modulated by maturity in many fruits such as strawberry [27], apple [28], litchi [29], kiwi [30] under natural conditions. In the present study, the expression patterns of six anthocyanin structural genes (*PbPAL*, *PbCHS*, *PbCHI*, *PbF3H*, *PbDFR*, *PbANS*) were basically consistent with the variation of anthocyanin content during fruit development, indicating that a synergistic and not the individual effect of these genes is responsible for anthocyanin biosynthesis and regulation. Meanwhile, the low expression level of *PbCHS*, *PbF3H*, *PbDFR*, *PbANS* 30 DAFB may be the result of a high anthocyanin content in a feedback regulatory loop (Figure 4). Tissue-specific expression analysis of anthocyanin biosynthetic genes showed that *PbPAL*, *PbCHS*, *PbCHI*, *PbF3H*, *PbDFR*, *PbANS* could be expressed in all tissues but at different levels. The structural genes for flower pigmentation in dicotyledonous plants can be divided into early biosynthetic genes (EBGs) for flavone and/or flavonol biosynthesis and late biosynthetic genes (LBGs), including *DFR* and *ANS*, for anthocyanin biosynthesis [31]. *PbF3H*, *PbDFR*, and *PbANS* were mainly expressed during the early flowering period, which explained the phenotype of reduced levels of anthocyanin content in petals (Figure 5).

Qian et al. [32] reported that reverse mutation caused color loss in the leaves and stems after grafting of 'Red Zaosu', but no fruit set in the mutated branches. Here, we obtained the mature fruit of the green mutant. The anthocyanin content in the green mutant was 10 times less than that in 'Red Zaosu' (Figure 6). Besides, the expression of LBGs such as *PbDFR* and *PbANS* and the transcription factor *PbMYB10* significantly decreased in the green mutant (Figure 7). The genes *DFR* and *ANS* in mildly colored pear 'Zaobaimi' (*Pyrus pyrifolia*) have been considered the limiting factors for peel coloration [33], while the transcript levels of *CHS*, *F3H*, *ANS*, and *UFGT* in the red Chinese sand pear 'Mantianhong' were very well correlated with anthocyanin levels in the pear skin [34]. *MYB10* has been widely reported to play a positive role in the regulation of anthocyanin biosynthesis by activating the structural genes in red pears and other Rosaceae plants [17,35,36]. However, the *MYB10* gene is not directly responsible for the difference in skin color between the red pear and the green mutant pear, although its expression level was significantly higher in the red pear [13,37]. Therefore, the expression patterns of *PbDFR*, *PbANS*, *PbMYB10* plays an important role in the coloration differences between 'Red Zaosu' and its green mutant. The function of *PbMYB10* requires further study.

5. Conclusions

Overall, the developmental changes in the anthocyanin profile observed in 'Red Zaosu' pear skin resulted from a synergistic effect of structural genes involved in the anthocyanin biosynthetic pathway. The levels of *PbF3H*, *PbDFR*, and *PbANS* were closely correlated with the variation of petal coloration during pear flower development. The decreased expression of *PbDFR*, *PbANS*, and *PbMYB10* may cause color loss in the fruit skin of 'Red Zaosu'.

Supplementary Materials: The following is available online at https://www.mdpi.com/article/10.3390/agriculture11090898/s1, Table S1: Primers used for qRT-PCR.

Author Contributions: Conceptualization, H.T. (Haoru Tang) and Y.Z. (Yong Zhang); methodology, X.G., D.L. and B.Z.; visualization, X.G., S.L. and H.T. (Honglan Tang); software, Y.W., M.L. and Q.C.; data curation, Y.Z. (Yunting Zhang); formal analysis, Y.Z. (Yunting Zhang) and Y.L. (Yuanxiu Lin); writing—original draft preparation, Y.Z. (Yunting Zhang) and S.L.; writing—review and editing, Y.L. (Ya Luo), X.W., H.T. (Haoru Tang) and Y.Z. (Yong Zhang). All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by grants from the Science and Technology Plan Project of Sichuan Province (Grant No. 2019YFN0122).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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