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Differential Coloration, Pigment Biosynthesis-Related Gene Expression, and Accumulation According to Developmental Stage in the 'Enbu' Apple

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Abstract: Coloration, a major factor contributing to apple quality, attracts consumer attraction, thereby increasing the sale of fruits in the market. Among the fruit pigments, anthocyanins and carotenoids are the most important pigments that impart coloration to apples. Pigment accumulation, which is regulated by transcriptional factors in the anthocyanin and carotenoid biosynthesis pathways, varies according to genetics, transcription, and developmental stage. To understand the regulation of color development in apples, we evaluated the expression of the genes in the anthocyanin and carotenoid biosynthetic pathways and analyzed the accumulation of pigment, including anthocyanin and carotenoid in the skin and flesh of the red-fleshed apple 'Enbu' cultivar at different fruit development stages (30, 60, 90, 120, and 150 days after full bloom, and ripe). Color development increased according to the developmental stage, with considerable variation in both the transcript levels and pigment concentrations observed in tissues, skin, and flesh. Moreover, we identified key transcription factors among the anthocyanin and carotenoid biosynthesis genes that regulated pigment accumulation. Pearson's correlation analysis showed a strong correlation between the coloration patterns and the expression levels of anthocyanin biosynthesis-related genes (*MdPAL*, *MdCHI*, and *MdF3H*) and carotenoid biosynthesis-related genes (*MdGGPPS*, *MdPSY*, *MdZDS*, *MdCRTISO*, *MdCRHβ*, and *MdZEP*). This study provides insight into the molecular mechanisms underlying pigment biosynthesis for breeding high-quality red-fleshed apple varieties to cater to consumer attention and preference.

Keywords: apple coloration; fruit quality; red-fleshed apple; pigment accumulation

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1. Introduction

While purchasing fruits, consumers are more likely to be attracted to fruits with rich natural colors, because they associate these colors with fruit properties such as taste, quality, and nutritional content [1,2]. By the same understanding, apples with deep coloration generally attract the attention of consumers because of their visual appeal in the market. Furthermore, fruit coloration is often a key indicator of ripeness; therefore, a rich and consistent color of apples suggests that the fruits are mature and ready to eat, which aligns with consumer expectations. In terms of quality and nutrition, apples with deep coloration often have high levels of antioxidants, which are beneficial to health, and are often perceived as fresh and more flavorful. Thus, owing to these factors, deeply colored apples are preferred by consumers more than apples that are lightly colored, thus increasing their sale prospects in the market.

Fruit coloration primarily arises from biochemical processes that encompass the synthesis of pigments, such as carotenoids and anthocyanins. The concentration of these pigments in the fruit dictates its color intensity. Hence, fruits with greater coloration are

associated with higher concentrations of pigments. The antioxidant properties of these pigments have significant health benefits [3–5]. Therefore, the utilization of these pigments in both food and nutritional supplements has been explored previously because of their biological activities [6,7].

In plants, the accumulation of anthocyanins and carotenoids occurs through two distinct biosynthesis pathways: the anthocyanin and carotenoid biosynthesis pathways. These pathways involve a sequence of enzyme-driven reactions encoded by their respective genes and have been comprehensively elucidated previously [8–11]. The process of anthocyanin biosynthesis commences with the transformation of the initial precursor, phenylalanine, into coumaroyl-CoA through the action of phenylalanine ammonia lyase (PAL). Subsequently, a series of enzymatic reactions involving chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) unfolds, ultimately resulting in the production of anthocyanidins. The produced anthocyanidin is further modified through processes such as glycosylation or acylation catalyzed by UDP-glucose: flavonoid glucosyltransferase (UFGT), resulting in the production of the final anthocyanin pigments. Contrastingly, the carotenoid biosynthetic pathway begins with the synthesis of geranylgeranyl diphosphate (GGPP) from isopentenyl pyrophosphate. Subsequently, GGPP is transformed into phytoenes by phytoene synthase (PSY). Throughout this pathway, a series of structural genes encode the enzymes responsible for catalyzing each step, ultimately leading to various downstream carotenoid products. MYB transcription factors are involved in anthocyanin accumulation [12–14]. They regulate the accumulation of anthocyanins through interactions with the MYB–bHLH–WD40 regulatory complex [15–17]. This interaction subsequently triggers the transcriptional activation of anthocyanin biosynthesis-related genes [18]. It is well known that the formation of pigments occurs through respective biosynthesis pathways under the regulation of genes encoding the enzymes involved in these pathways. Therefore, these transcription factors have been widely used as marker genes for evaluation of pigment accumulation related to their expression profiles in apples [8,19–21]. Upregulation of these transcription factors by overexpressing induced the accumulation of pigment in apples [11,22–24]. Recent studies identify their function in improving the nutritional value (β-carotene is a precursor to vitamin A) in different crops through metabolic engineering approaches to the phytoene synthase gene [25,26].Changes in coloration related to the pigment accumulation in apples can be affected by environmental stimuli, such as light [23,27–29] and temperature [30,31]. Generally, these changes are variable and dependent on different developmental stages of the fruit and normally increase with respect to the stage of harvest by maturity [8]. A similar phenomenon has been reported in pears [32,33]. These studies show that the differential expression of anthocyanin biosynthesis genes and their accumulation changed the coloration at different fruit development stages and between different cultivars. Such color transformation is a visually striking aspect that reflects the underlying biochemical and physiological changes occurring in the fruit.

The red-fleshed apple cultivar 'Enbu', which was recently developed by the Apple Research Institute, National Institute of Horticultural and Herbal Science, Rural Development Administration, is expected to be widely planted in orchards to contribute to the increasingly developing national apple industry. The fruit of this cultivar has red flesh, which may attract consumer interest. During field trials, the skin of the 'Enbu' apple was found to turn red at the middle stage of fruit development. However, the flesh did not turn red during the same period as that of the skin, until ripening. This suggests that color development in the skin and flesh of 'Enbu' apples follows different trends and depends on the molecular mechanism of the pigments in these tissue types. The 'Enbu' apple is a newly developed cultivar showing distinctive color development, but it has not been investigated. The present study was conducted to investigate the differences in coloration, biosynthesis gene expression, and pigment accumulation in the skin and flesh of apples during fruit development. By systematically analyzing the expression of genes involved in anthocyanin and carotenoid biosynthetic pathways and quantifying the accumulation of

these pigments in relation to fruit coloration patterns, we aimed to identify key genes that control coloration in both the skin and flesh tissues, according to the developmental stages of the red-fleshed 'Enbu' apple cultivar. Additionally, the present study was conducted to investigate whether the different trends in color development between the skin and flesh of 'Enbu' apples depend on the molecular mechanism of pigments. Factors that determine fruit quality attributes during fruit development, including soluble solid content (SSC), titratable acidity (TA), firmness, and starch index (SI), were also investigated. Overall, this study provides comprehensive insights into the molecular mechanisms of pigment accumulation that drive these color changes and how they correlate with different stages of fruit development in red-fleshed apples.

2. Materials and Methods

2.1. Fruit Materials

The fruits used in this study were collected from 4-year-old 'Enbu' apple trees (grafted to M.9 rootstock) grown in the experimental field of the Apple Research Institute in Gunwi (36.28 N, 128.47 E), South Korea, in 2022. The trees were gown in the experimental field trial, with weather conditions as shown in Figure 1. The fruit of the 'Enbu' apple cultivar is red skinned and red fleshed. It flowers in full bloom in late April (22 April), and its fruit is harvested in late October. For setting the timepoint of the fruit sample date, the first day of full bloom was recorded and is shown in Figure 2. Apple fruits were sampled at different development stages, namely, 30, 60, 90, 120, and 150 days after full bloom (DAFB) and at the ripening stage. At each developmental stage, 15 fruits were randomly collected from several individual trees (3 fruits per tree) with triplicates per timepoint. They were used for the assessment of fruit development (weight and size), quality attributes, and coloration. The skin and flesh tissues of fruits were sampled, rapidly frozen in liquid nitrogen, and preserved at −80 °C for subsequent analyses, including RNA isolation and pigment content extraction.

2.2. Measurement of Fruit Color and Assessment of Fruit Quality Attributes

The collected fruits were used to evaluate fruit development by measuring the weight, size, and color of fruits and assess the fruit quality attributes. For fruit coloration, the skin of the fruits was measured at three random positions using a chroma meter (CR-400, Konica Minolta, Tokyo, Japan). The value of the color indices is expressed as a Hunter value (L, a, and b) [34]. Fruit firmness was measured at three random positions after the fruit skin was peeled off using a firmness tester (TR Turoni, Forlì, Italy) equipped with a Ø8 mm plunger. The extracted fruit juice was used to measure the titratable acidity (TA) and soluble solid content (SSC). SSC was measured using a pocket refractometer (PAL-1; Atago, Tokyo, Japan). TA was determined by titration using 0.1 N NaOH, with the results expressed in terms of malic acid equivalents. For the starch index (SI), fruits were cut in half by horizontal diminution; subsequently, an iodine solution was applied to the cut-off surface of the fruit for 5 min. SI was determined based on a score chart (Scores 1–8) [35]. Color index (CI) was calculated as follows [36]:

$$CI = \frac{180 - h}{C + L}$$

where h represents the hue angle calculated according to the following equations: $h = \tan -1(a/b)/6.2823 * 360$ when $a \geq 0$ and b ≥ 0 or $h = \tan -1(a/b)/(6.2823 * 360 + 180)$ when $a < 0$ and $b > 0$, and C represents chroma calculated using the following formula:

$$C = \sqrt{a^2 + b^2}$$

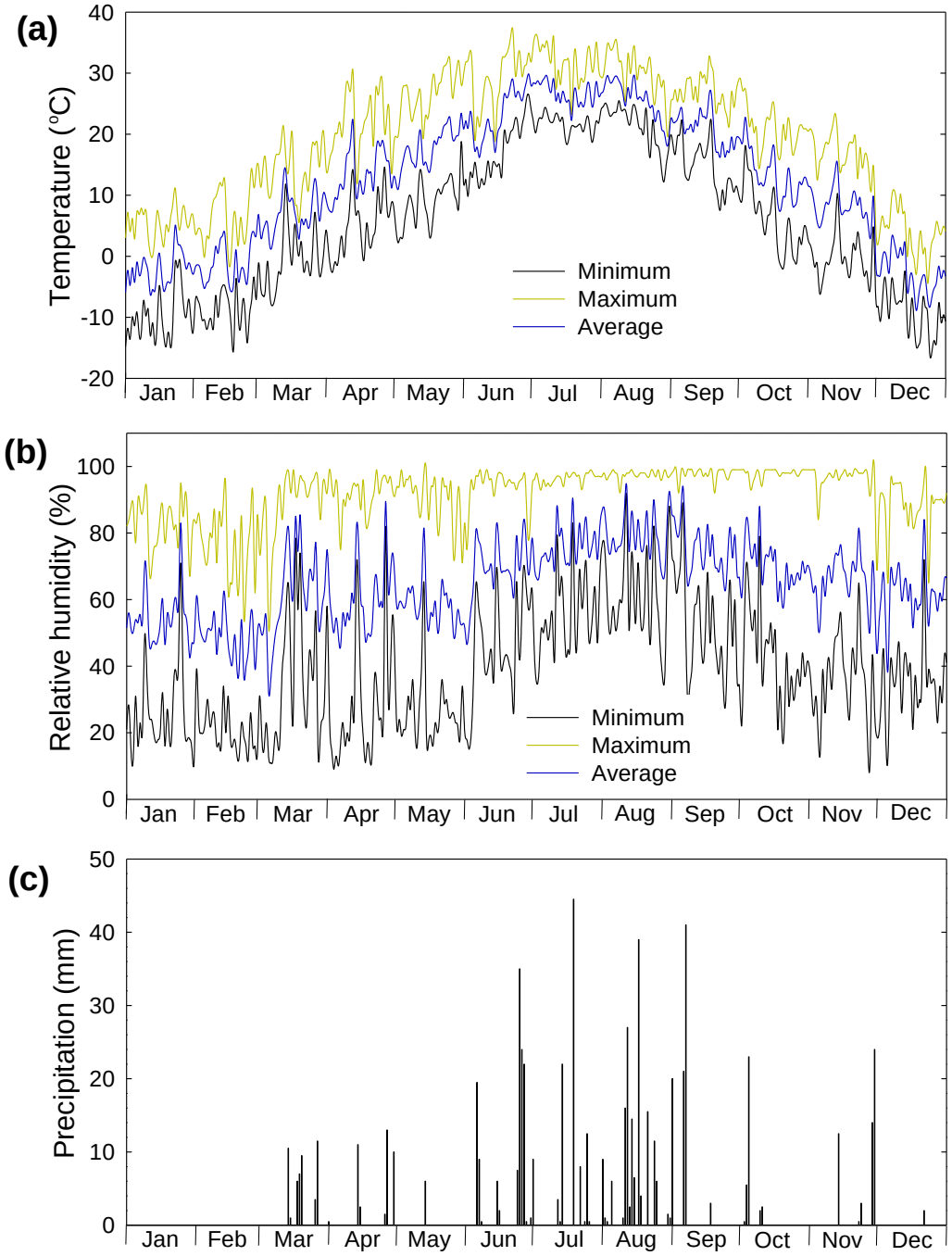


Figure 1. Meteorological data for 2022 for the experimental field trial of the Apple Research Institute in Gunwi South Korea. Change in (**a**) daily temperature, (**b**) daily relative humidity (rainfall), and (**c**) daily precipitation.

2.3. RNA Extraction and Quantification of Gene Expression

The sampled skin and flesh tissues of the apples were used to isolate total RNA using the cetyltrimethyl ammonium bromide (CTAB) method [37]. Before the synthesis of cDNA, the RNA samples were treated with DNase to remove genomic DNA contamination. The quality and concentration of isolated RNA was measured using an ultraviolet spectrophotometer. First-strand cDNA was synthesized from 1.0 µg total RNA using a PrimeScript™ 1st strand cDNA Synthesis Kit with the oligo dT primer (Takara, Kusatsu, Japan).

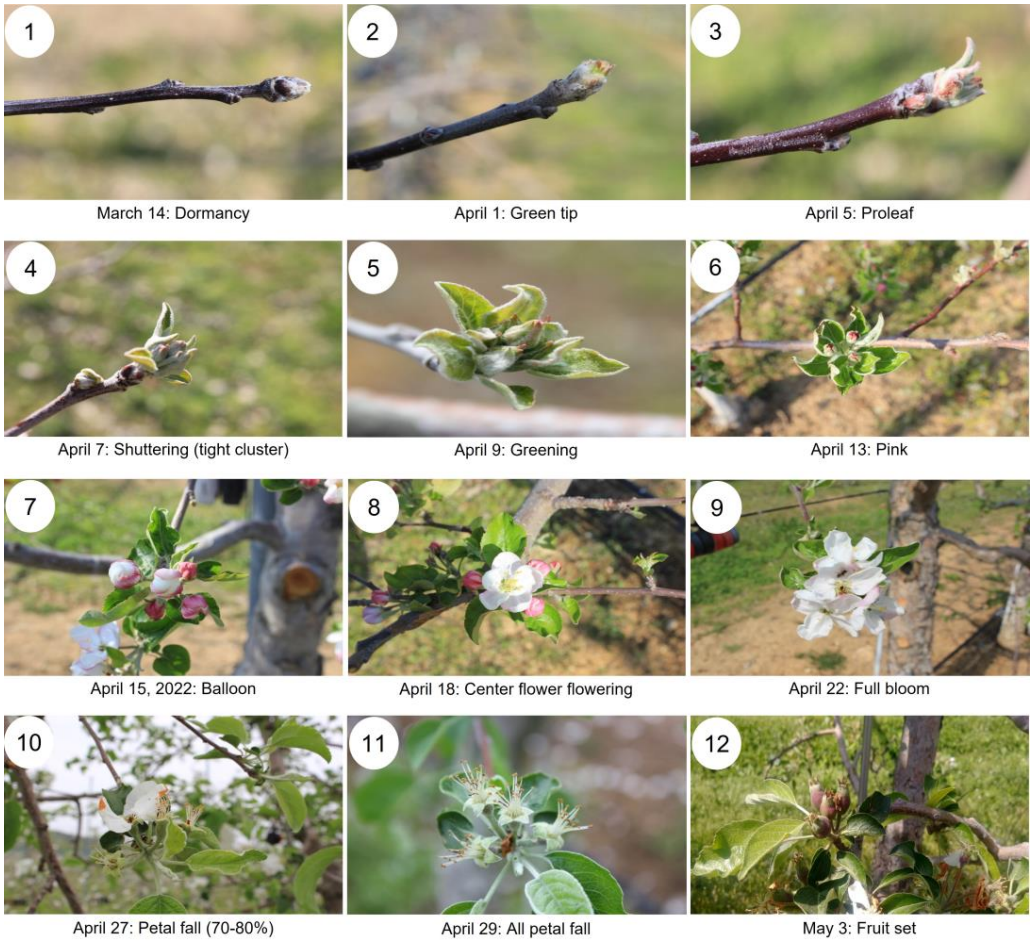


Figure 2. Phenological changes of the flower according to the development stages of the 'Enbu' apple in 2022. The 1st day of full bloom was recorded on 22 April, when the 'Enbu' apple was 70–80% bloomed. (**1**) Domancy; (**2**) Germination period: the tip of a green leaf containing a berry inside is visible; (**3**) Proleaf stage: the stage when the flower shoots spread out, and the green leaves have grown up to 10mm in scale; (**4**) Shuttering: flower buds are formed; (**5**) Greening stage: the period when flower buds are covered with calyx pieces and turn green. (**6**) Pink: the petals are exposed outside the calyx and turn pink, and the petals are observed; (**7**) Balloon stage: the period when the petals grow and become balloon-shaped; (**8**) Center flower flowering: the first flower (center flower) blooms; (**9**) Full bloom: 70–80% bloomed; (**10**) Petal fall (falling flower): 70–80% of the flowers have fallen; (**11**) Flowers have fallen: all petals have fallen; (**12**) Fruit set: the size of the fruit is 10mm in diameter.

Gene expression was evaluated using quantitative real-time–polymerase chain reaction (qRT-PCR), which was performed on a LightCycler 480 II Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). qRT–PCR was conducted in 20 μL of reaction volume, including 5 μL of cDNA (50 μg), 1μL of each (forward and reverse) primer (0.5 μM), and 3 μL of RNase-free water in 10μL of LightCycler 480 SYBR Green I Master Mix (Roche, Basel, Switzerland). qRT-PCR was run with the conditions as described in a previous study [29]. Briefly, qRT-PCR was performed on 96-well plates (LightCycler 480 Multiwell Plate 96, Roche) with running conditions as follows: 95 °C for 5 min, followed by 40 repeated cycles (95 °C for 10 s, 60 °C for 15 s, and 72 °C for 12 s); melting curve analysis was performed at 65–95 °C. qRT-PCR analyses were run in three biological replicates, with seven technical replicates per each biological replicate. cDNA-free templates were run along with the sample as controls for the confirmation of any potential sample contamination. The expression level of the target genes was quantified as their transcript levels normalized to the transcript levels of the reference gene *MDP0000336547*

(an apple SGF 29 tudor-like domain-containing protein) [38]. The relative expression levels of targets were calculated using the Roche® 480 Light Cycler software (version 1.5.1, Roche) E-Method. The primer sequences used for the qRT-PCR of the anthocyanin and carotenoid biosynthesis genes are listed in Tables S1 and S2, respectively.

2.4. Quantification of Pigment Contents

The ground powder of frozen apple skin and flesh samples was used for the quantification of pigment content. Anthocyanin concentration was quantified using the pH differential method [39]. Briefly, a specific quantity of the homogenized sample was mixed with 25 mL of methanol containing 1% HCl. The mixture was agitated in the dark at 4 °C for 20 min. Following this, the homogenates were sonicated and centrifuged (4000 rpm for 10 min), and the resulting supernatant was collected separately. Solutions with pH values of 1.0 and 4.5 were added to pre-determined volumes of this supernatant and stirred. Subsequently, the absorbance was measured at wavelengths of 520 and 700 nm. Anthocyanin concentration was quantified as an equivalent of cyanidin-3-glucoside, with an extinction coefficient of 26,900 L/cm. mol and a molecular weight of 449.2 g/mol.

The carotenoid content was quantified using a high-performance liquid chromatography (HPLC) system according to the method described previously [11]. A certain amount of the ground sample was suspended in 3% pyrogallol and 60% KOH. This mixture was then subjected to saponification by incubating at 70 °C, followed by a cooling step. After adding a solution containing 1% NaCl and a mixture of ethyl acetate and hexane, the samples were homogenized by vortexing. The resulting mixture was centrifuged, and the supernatant was collected. The supernatant was re-extracted by repeating this process several times, until it became clear. It was then concentrated using nitrogen gas before dissolution in ethanol. This solution was used as an analytical sample for carotenoid assessment via HPLC. Further, for HPLC analysis, 10 µL of the sample was injected into the system, using the Shiseido UG 120 column. Carotenoid separation was achieved using a mobile phase comprising methanol, acetonitrile, and dichloromethane at a flow rate of 1 mL/min. Carotenoids were determined at 450 nm, and carotenoid concentrations were quantified as equivalents of β-carotene per gram of fresh tissue.

2.5. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (IBM Corp., Armonk, NY, USA) and Microsoft Excel (Microsoft Corp., Redmond, WA, USA). The Pearson's correlation coefficient (r) test was performed to evaluate the relationship between coloration patterns, gene expressions, and anthocyanin contents. Data are expressed as mean ± standard deviation (SD) from triplicate experiments. Significant differences among the groups were determined using Tukey's honest significant difference (HSD) test ($p < 0.05$).

3. Results

3.1. Fruit Enlargement and Quality Attributes According to Developmental Stages

Fruit development is a complex process characterized by various stages, each marked by specific physiological and morphological changes. The enlargement of apples during these developmental stages is a crucial aspect affecting the size, quality, and overall commercial value of fruits. Fruit enlargement was evaluated at different stages of fruit development. Apples were collected every 30 days to measure their weight and size. Fruit enlargement changed at different stages of fruit development (Figure 3). Apples grew quickly, with a rapid increase in fruit size at the early developmental stage (30–90 DAFB), and then tended to slowly increase from 120 DAFB to the ripe stage (Figure 3a,d). In correlation with changes in the fruit weight, fruit size also rapidly increased at the early developmental stage (20.45 ± 3.52 at 30 DAFB to 307.60 ± 16.56 g at 120 DAFB) and slowly increased subsequently (Figure 3b). There were no differences in fruit shape during the different stages of fruit development, except at 30 DAFB. The ratio of length to diameter (L/D) changed marginally (Figure 3c). The slightly higher L/D value at 30 DAFB may be

caused by the initial stages of fruit development (after fertilization to 30 DAFB); further, the fruit was small, and the shape was not well-defined because the rate of cell division was predominant.

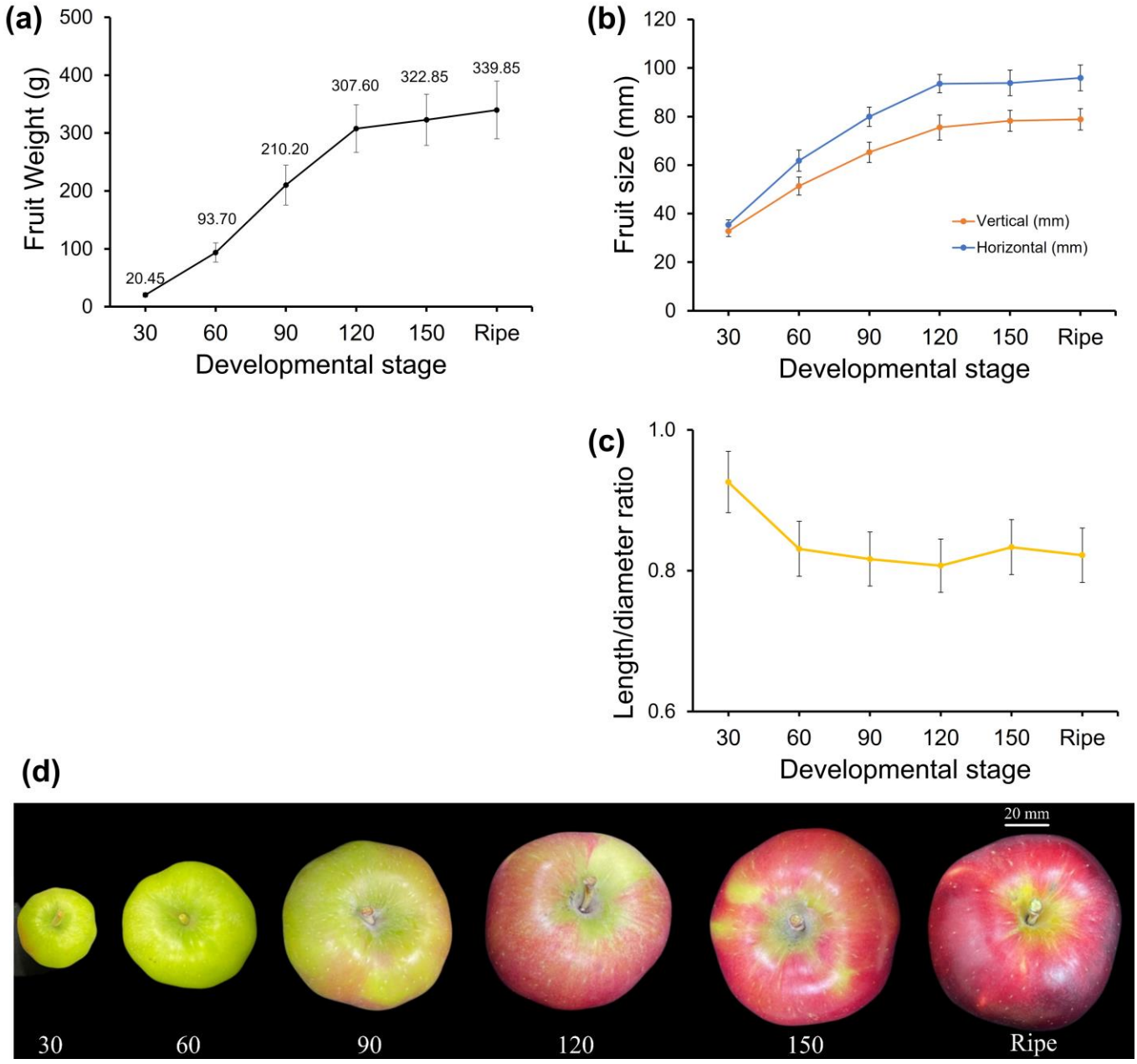


Figure 3. Changes in fruit enlargement at different fruit development stages: (**a**) fruit weight, (**b**) fruit size (vertical and horizontal diameter), and (**c**) ratio of length to diameter. (**d**) Morphology of 'Enbu' apples during development and ripening stages. Bars = 20 mm. Data are presented as mean ± standard deviation (SD) (n = 15). Fruits were sampled at different development stages with the respective dates, as follows: 30 DAFB (23 May), 60 DAFB (22 June), 90 DAFB (22 July), 120 DAFB (22 August), 150 DAFB (22 September), and at the ripening stage (25 October).

The quality of apples is influenced by various attributes, such as taste, texture, and nutritional content. Along with the overall appearance, we evaluated the SSC, TA, firmness, and SI. These fruit quality attributes were examined from 90 DAFB to the ripe stage, as shown in Table 1. The attributes changed significantly as the fruit progressed through the different developmental stages. In general, the firmness index, TA, and SI showed a

decreasing tendency, while SSC showed an increasing tendency and reached the highest value (14.7 ± 0.7 °Brix) at the ripe stage.

Table 1. Fruit quality attributes during the development process in 'Enbu' apples.

Developmental Stage	Firmness (N)	SSC (°Brix)	TA (%)	SI (Scores 1–8)
90 DAFB	88.68 ± 2.77 [a]	10.8 ± 0.5 [d]	2.29 ± 0.23 [a]	6
120 DAFB	83.69 ± 4.36 [b]	12.7 ± 0.4 [c]	2.06 ± 0.27 [b]	5
150 DAFB	71.57 ± 3.15 [c]	13.7 ± 0.6 [b]	1.50 ± 0.18 [c]	3
Ripe	59.59 ± 3.52 [d]	14.7 ± 0.7 [c]	1.11 ± 0.12 [d]	1

All values are shown as mean ± SD of 15 individual fruits. Distinct letters indicate statistically significant differences as determined by Tukey's HSD test ($p < 0.05$).

3.2. Fruit Coloration Patterns during Fruit Development

Skin color properties and development were measured during different fruit developmental stages (Figure 4). The color patterns of the fruits were evaluated using Hunter values (*L*, *a*, and *b*) (Figure 4a) and expressed as the color index (Figure 4b). The *a* value exhibited a positive correlation with the degree of fruit coloration, whereas the *L* and *b* values showed a negative correlation with the level of fruit coloration. At the early developmental stages (30–90 DAFB), fruit coloration was green, with a negative *a* value and high *L* and *b* values (Figure 4a). Subsequently, the fruit quickly turned red, especially during 90–120 DAFB (Figure 4c). At the late fruit developmental stage (120–150 DAFB) and ripening stage, the fruit skin color intensity increased and reached the highest levels at the ripening stage. Apparent differences in coloration patterns in the later stages correlated with skin color properties. When the fruit color turned red at 90–120 DAFB, the *a* value (denoted by red) increased from a negative value (−15.66 at 90 DAFB) to a positive value (19.20 at 120 DAFB). Additionally, *L* and *b* changed from a high value to a low value; that is, they decreased from 51.37 to 35.86 and 23.28 to 6.61, respectively, and remained relatively stable during the late stages of fruit development (120–150 DAFB and the ripe stage), as the apparent coloration patterns changed only slightly. Regarding the Hunter value, the color index showed a similar trend, with a positive correlation with the red coloration level (*a* value) and a negative correlation with *L* and *b* values. At the early fruit development stages, the color index value was low (0.67–0.70) for 30–90 DAFB; subsequently, the color index showed a higher value (2.87) at 120 DAFB, when the fruit skin turned red. The color index value slowly increased and reached the highest value (3.09) during the ripening stage (Figure 4b). In general, the coloration levels of the fruit skin changed across developmental stages, in correlation with changes in the Hunter value and color index. Furthermore, in addition to the coloration patterns of the skin, we observed changes in the flesh of the fruit during fruit development (Figure 4d). Interestingly, unlike the skin, the fruit flesh showed a different coloration pattern. The skin of the fruit turned red at 90 DAFB, whereas the fruit flesh was still white until the late developmental stage (150 DAFB), and finally, it turned red at the ripening stage. Furthermore, the apparent coloration patterns differed between the fruit skin and flesh.

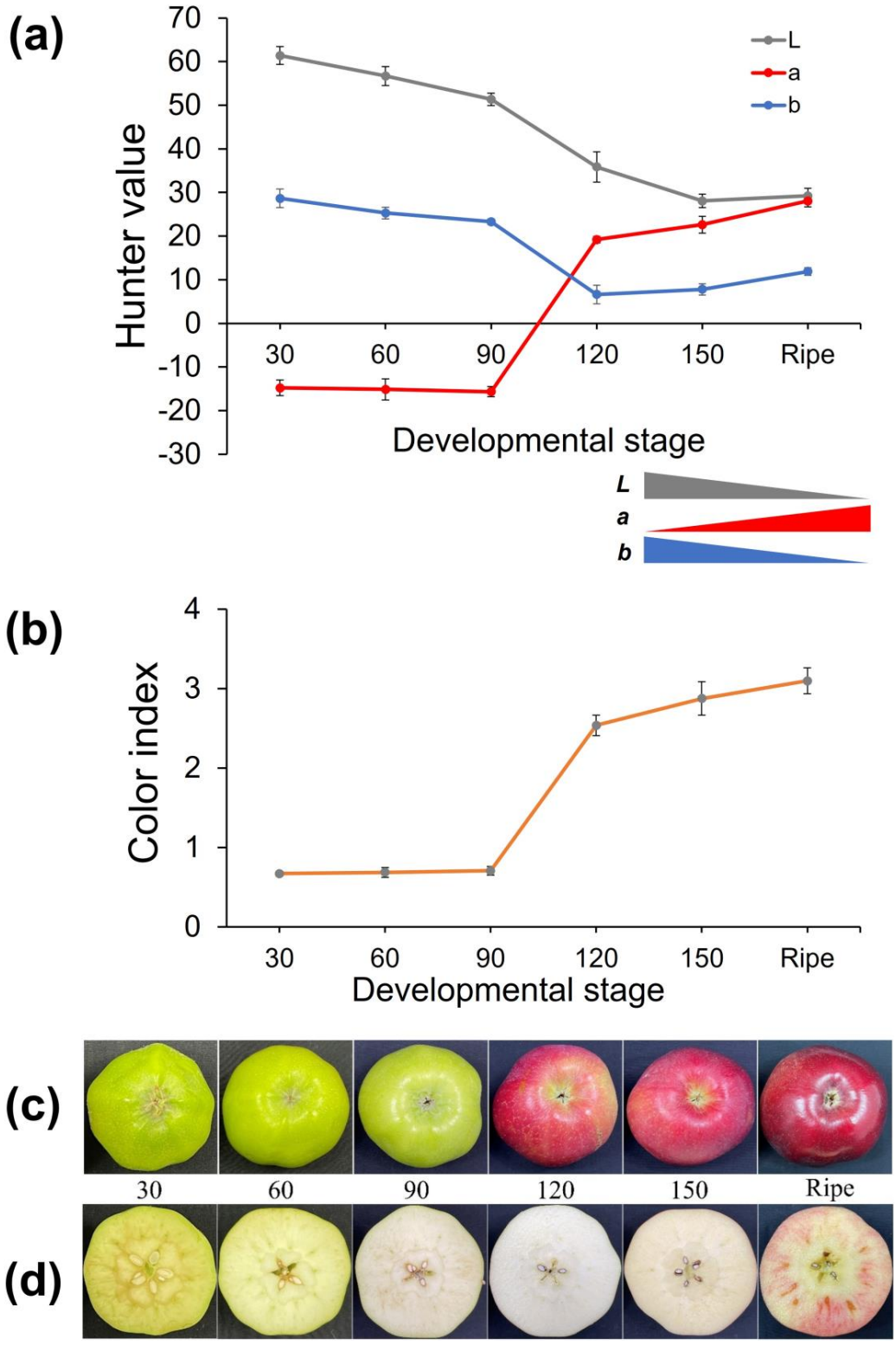
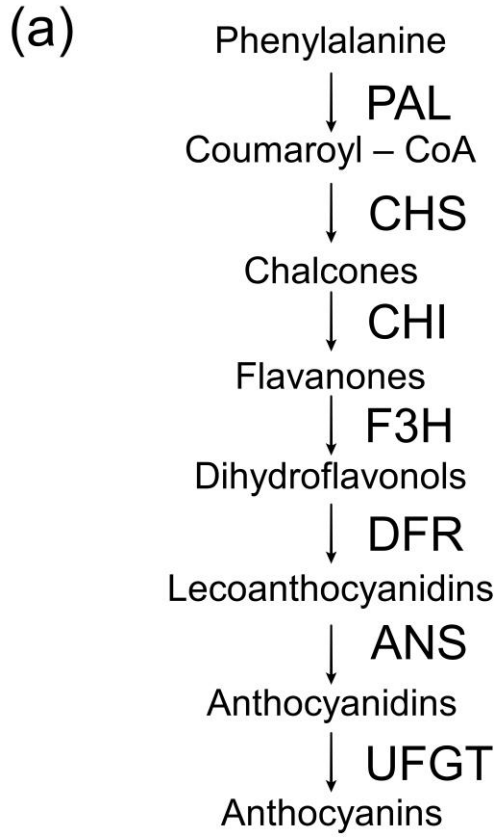


Figure 4. Coloration patterns of 'Enbu' apples at different fruit development stages. (**a**) Color indices (*L*, *a*, and *b*) and (**b**) color index of the apple skin according to developmental stages. (**c**,**d**) Differential coloration patterns in fruit skin (**c**) and flesh (**d**) at different fruit development stages. During the fruit development process, fruits are represented by immature green (30–60 DAFB), mature green (90 DAFB), turning red (120 DAFB), mature red (150 DAFB), and full red (ripe). *L*: light—dark scale. *a*: green—red scale. *b*: yellow—blue scale.

3.3. Expression Profiles of the Anthocyanin Biosynthesis-Related Genes

The synthesis of anthocyanin pigments, which are responsible for the coloration of apples, is a complex process regulated by a series of genes. To determine the expression profiles of genes responsive to fruit coloration patterns during fruit development, we evaluated the transcript levels of the anthocyanin biosynthesis-related genes (Figure 5a) according to developmental stages via qRT-PCR (Figure 5b). The expression of these genes varied across the different developmental stages of the apples, in both skin and flesh tissues. In the skin tissues, most anthocyanin synthesis-related structural genes showed high expression in the late stages (150 DAFB and ripe stage). Some of these (*MdCHS*, *MdDFR*, *MdANS*, and *MdUFGT*) were also highly expressed in the early stage (30 DAFB), whereas they were weakly expressed at the stages of 60–120 DAFB. The expression of *PAL* and *CHS* increased during fruit development. Similar to skin tissues, most anthocyanin synthesis-related structural genes were highly expressed in the early (30 DAFB) and late stages (ripe stage), except *MdPAL*. The expression profiles of *MdCHS*, *MdF3H*, *MdDFR*, *MdANS*, and *MdUFGT* were similar. They were strongly expressed in the early stage (30 DAFB), decreased to a low expression level (60–150 DAFB), and then increased at the ripe stage. In addition to the anthocyanin synthesis-related structural genes, the transcription factor *MdMYB10* showed similar expression patterns in skin and flesh tissues. In both tissues, the expression levels of *MdMYB10* were increased gradually from 30 to 90 DAFB and decreased rapidly thereafter. The expression profiles of anthocyanin synthesis-related structural genes were compared between the skin and flesh tissues. We found that the expression of these genes was higher in the skin than in the flesh during development, except for *MdCHI* and *MdUFGT*, the expression of which was higher in the flesh than in the skin at the early (30 DAFB) and ripening stages.



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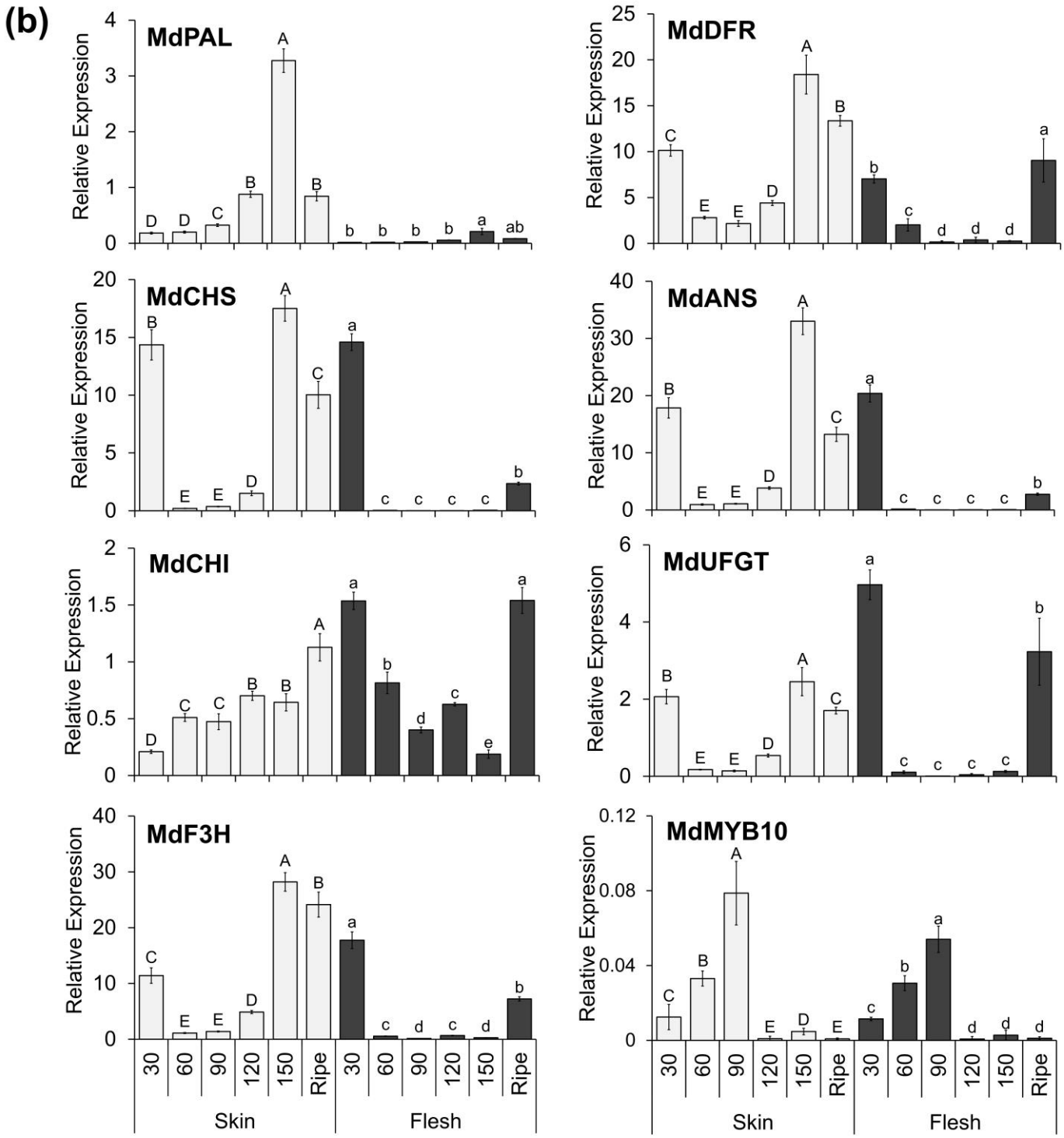
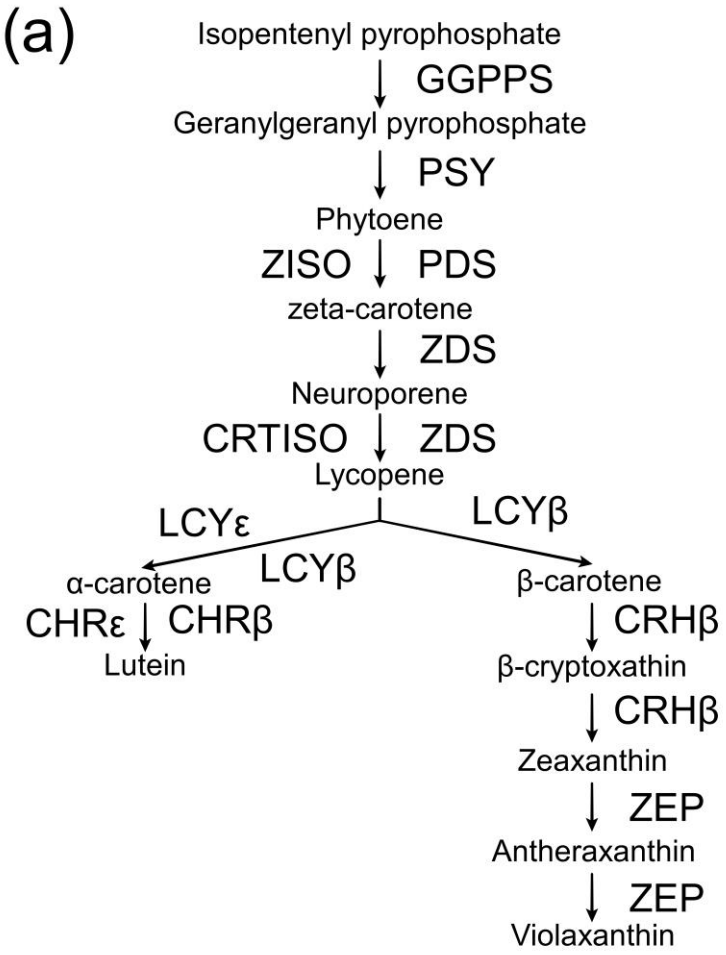


Figure 5. (**a**) Anthocyanin biosynthesis pathway. (**b**) Expression profiles of anthocyanin synthesis-related structural genes (*MdPAL*, *MdCHS*, *MdCHI*, *MdF3H*, *MdDFR*, *MdANS*, and *MdUFGT*) and transcription factor (*MYB10*) in the skin and flesh tissues of 'Enbu' apples according to developmental stages. Transcript levels were calculated in normalization to an apple SGF 29 tudor-like domain-containing protein (*MDP0000336547*) gene. Data are expressed as means ± SD of three biological replications. Different uppercase letters denote a significant difference ($p < 0.05$) in the skin, and different lowercase letters denote a significant difference ($p < 0.05$) in the flesh.

3.4. Expression Profiles of the Carotenogenic Genes

To evaluate the expression levels of genes involved in the regulation of carotenoid synthesis according to developmental stage, the expression levels of carotenoid biosynthesis-related genes (Figure 6a) were determined using qRT-PCR (Figure 6b). During fruit development, their transcript levels varied in both the skin and flesh. Similar to that of the transcript levels of anthocyanin biosynthesis-related genes, the expression of the carotenoid biosynthesis-related genes showed high expression at late stages (150 DAFB and ripe stage) in the skin, except *MdPDS* and *MdLCYε*. In the flesh, the expression of carotenoid biosynthesis-related genes was also high in the late stages (150 DAFB and ripe stage). However, the expression level of *MdLCYε* was an exception. Its expression showed high levels at the early stages (30–60 DAFB) but was low later. A similar trend was observed in the skin, with the highest expression levels observed during the early stages. In the comparison between skin and flesh, the expression level of *MdGGPPS*, *MdLCYε*, and *MdZEP* in the skin was higher than that in the flesh at all developmental stages. However, the relative expression level of other genes (*MdPSY*, *MdPDS*, *MdZISO*, *MdZDS*, *MdCRTISO*, *MdLCYβ*, and *MdCRHβ*) was higher in the flesh than in the skin at the late fruit development stage (150 DAFB). In the flesh, the expression of these genes remained low at 30–120 DAFB, rapidly increased, peaked at the highest level at 150 DAFB, and then decreased at the ripe stage. The expression levels of *MdGGPPS* increased gradually during fruit development in the skin but did not change much in the flesh.



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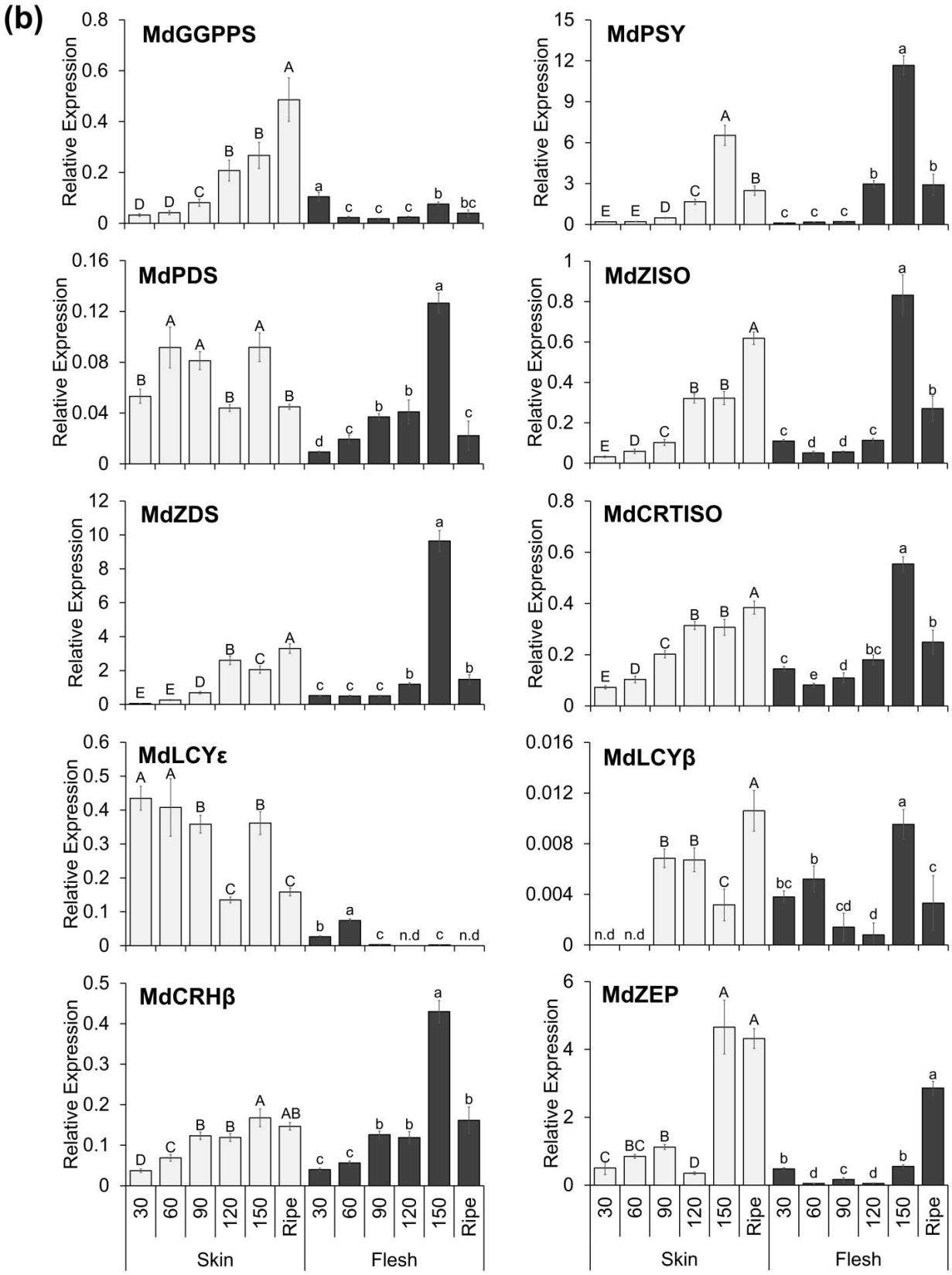

Figure 6. (**a**) Carotenoid biosynthesis pathway. (**b**) Relative expression profiles of carotenoid biosynthesis genes according to developmental stages in the skin and flesh tissues of 'Enbu' apples.

MdGGPPS, geranylgeranyl pyrophosphate synthase; *MdPSY*, phytoene synthase; *MdPDS*, phytoene desaturase; *MdZISO*, zeta-carotene isomerase; *MdZDS*, zeta-carotene desaturase; *MdCRTISO*, carotenoid prolycopene isomerase; *MdLCYε*, epsilon lycopene cyclase; *MdLCYβ*, lycopene beta cyclase; *MdCRHβ*, beta-carotene hydroxylase 2; and *MdZEP*, zeaxanthin epoxidase. qRT-PCR assays were conducted in three biological replications. Transcript levels were calculated in normalization to an apple SGF 29 tudor-like domain-containing protein (*MDP0000336547*) gene. Data are expressed as mean ± SD. Different uppercase letters denote a significant difference ($p < 0.05$) in the skin, and different lowercase letters denote a significant difference ($p < 0.05$) in the flesh. n.d refers to non-detectable.

3.5. Accumulation of Pigment Content

The accumulation of pigments is crucial in defining both the color and nutritional composition of apples. Changes in pigment accumulation through different developmental stages of apples are key indicators of fruit maturation and quality. The accumulation of pigment, including anthocyanin and β-carotene, was analyzed in both the skin and flesh of fruits. Changes in anthocyanin accumulation according to the developmental stage are shown in Figure 7. In general, anthocyanin accumulation showed an increasing trend during fruit development in both the skin and flesh. In the skin tissues, anthocyanin accumulation was low during the early stages of fruit development (30–60 DAFB), increased (90–120 DAFB), and reached its highest content (155.61 μg/g) at the ripe stage of fresh weight (FW) (Figure 7a). Similar to the skin tissues, the flesh tissues showed low accumulation of anthocyanins at the early stages of fruit development, with no significant difference at these stages. At mid-development, anthocyanin accumulation increased at 90–120 DAFB, remained the same at 120–150 DAFB, and dramatically increased to the highest amount at the ripe stage (4.46 μg/g of FW) (Figure 7b). Additionally, in the comparison between skin and flesh, the anthocyanin content in the skin was much higher than that in the flesh at all stages of fruit development.

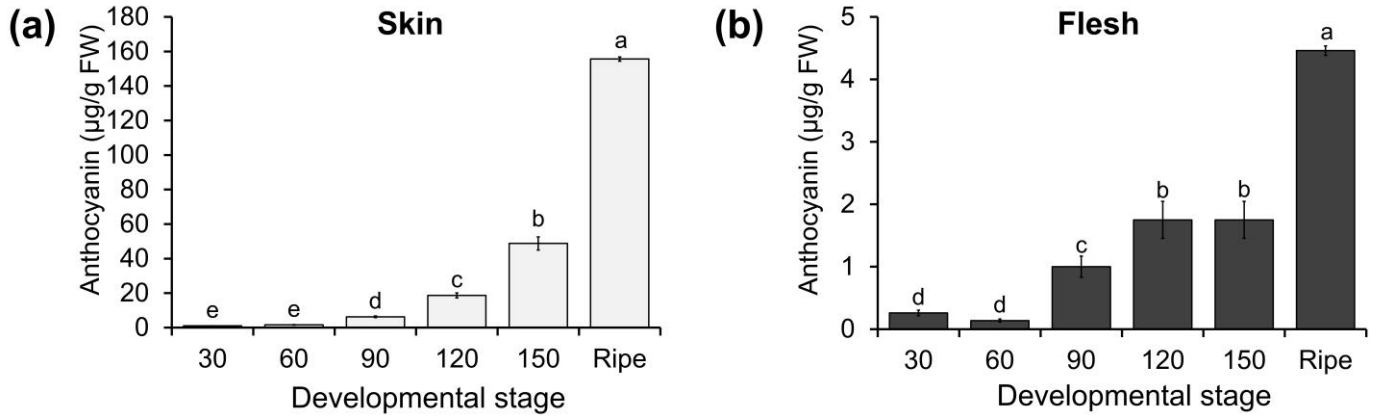

Figure 7. Accumulation of anthocyanin in the skin (**a**) and flesh (**b**) of 'Enbu' apples at different fruit development stages. Data are expressed as the mean ± SD ($n = 3$). Different letters represent a significant difference ($p < 0.05$).

The accumulation of β-carotene during fruit development is shown in Figure 8. In the skin tissues, β-carotene contents were the highest at the early stages of fruit development (30 DAFB), decreased (60–120 DAFB), and then increased again at the late stages (150 DAFB to the ripe stage) (Figure 8a). In the flesh tissues, the accumulation of β-carotene was high in the early stages (30 DAFB). Subsequently, β-carotene concentration declined (60–90 DAFB), accumulated the same amount at 120 and 150 DAFB, and then slightly dropped at the ripe stage (Figure 8b).

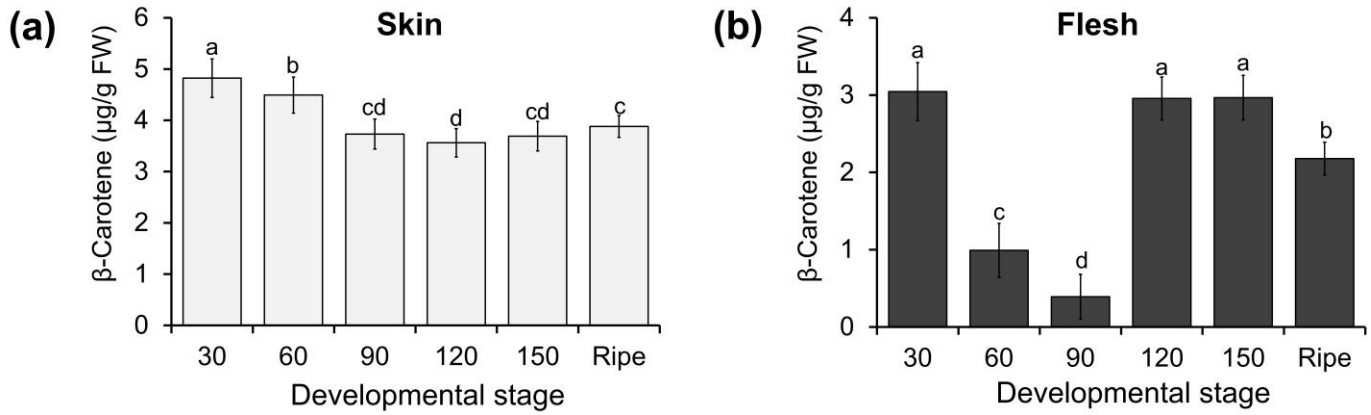

Figure 8. Concentration of β-carotene in the skin (**a**) and flesh (**b**) of 'Enbu' apples at different fruit development stages. Data are expressed as means ± SD ($n = 3$). Different letters represent a significant difference ($p < 0.05$).

3.6. Correlation Analysis of Coloration Patterns, Gene Expression Levels, and Pigment Contents

Pearson's correlation coefficient analysis was performed to elucidate the relationship between gene expression levels, coloration patterns (color index), and pigment accumulation. Among the anthocyanin biosynthesis-related genes (Table 2), the expression of *MdPAL*, *MdCHI*, and *MdF3H* showed a strong correlation with coloration patterns. The expression of the remaining genes moderately correlated with coloration. Correlation analysis of gene expression levels and anthocyanin content showed that *MdCHI* was strongly correlated with anthocyanin content. The expression levels of *MdF3H* showed a strong correlation, *MdDFR* showed an intermediate correlation, and the other genes showed a weak correlation. For carotenoids (Table 3), the correlation analysis revealed a positive correlation, ranging from strong to very strong, between the coloration patterns and the expression levels of genes associated with carotenoids (*MdGGPPS*, *MdPSY*, *MdZDS*, *MdCRTISO*, *MdCRHβ*, and *MdZEP*), but *MdPDS* and *MdLCYε* were negatively correlated. Further, β-carotene contents and gene expression showed a strong correlation with *MdGGPPS* but an intermediate to weak correlation for other genes. *MdLCYε* was also negatively correlated to β-carotene contents. Additionally, anthocyanin contents and coloration patterns were strongly correlated, but moderate correlation was observed between β-carotene contents and coloration patterns.

Table 2. Pearson's correlation coefficient (*r*) analysis of anthocyanin-related gene expression levels, coloration patterns, and anthocyanin accumulation.

Correlation Coefficient (*r*)	Gene Expression							
	MdPAL	**MdCHS**	**MdCHI**	**MdF3H**	**MdDFR**	**MdANS**	**MdUFGT**	**Anthocyanin**
Color Index	0.745	0.426	0.759	0.760	0.681	0.538	0.495	0.681
Anthocyanin	0.252	0.331	0.897 *	0.707	0.563	0.282	0.408	1.000

* Level of significance: $p < 0.05$. The correlation coefficient (*r*), varying in the range of 0 to 1.0, refers to the level of correlation from weak to strong, respectively.

Table 3. Pearson's correlation coefficient (*r*) analysis of carotenoid biosynthesis gene expression levels, coloration patterns, and β-carotene accumulation.

Correlation Coefficient (*r*)	Gene Expression										
	MdGGPPS	**MdPSY**	**MdPDS**	**MdZISO**	**MdZDS**	**MdCRTISO**	**MdLCYε**	**MdLCYβ**	**MdCRHβ**	**MdZEP**	**β-Carotene**
Color Index	0.858 *	0.816 *	−0.263	0.869 *	0.920 **	0.908 *	−0.685	0.555	0.807 *	0.920 **	0.569
β-Carotene	0.674	0.497	0.302	0.469	0.423	0.533	−0.351	0.273	0.318	0.146	1.000

Levels of significance: * $p < 0.05$; ** $p < 0.01$. The correlation coefficient (*r*), varying in the range of 0 to 1.0, refers to the level of correlation from weak to strong, respectively.

4. Discussion

4.1. Changes in Coloration Patterns According to the Fruit Development Stages

Color is one of the most important traits in fruit development and ripening. The coloration patterns of apples undergo distinct changes throughout fruit development. These changes are primarily driven by changes in pigmentation, cell structure, and chemical composition [40]. The transformation in color not only affects the aesthetic appeal of the fruit but also provides crucial information about its ripeness and quality [41]. In this study, we evaluated changes in coloration patterns during fruit development (Figure 4). Additionally, the changes in fruit enlargement, including weight, size, and shape, were evaluated (Figure 3). During the early stages of apple fruit development (30 DAFB), the predominant fruit color was green, arising from the presence of chlorophyll, a pigment responsible for photosynthesis. As the fruit grew, it underwent cell division and expansion, leading to increases in size and weight. At this stage of fruit development, the rate of cell division was predominant, resulting in a relatively rapid increase in fruit size; thus, the fruit shape was not well-defined. The L/D ratio was greater in the early stages than that in the later stages (Figure 3c). After the apples transitioned into the mid-developmental stages, cell expansion continued to become more prominent. The fruits exhibited significant growth in both size and volume (60–120 DAFB) (Figure 3a,b,d). The cells in the fruit flesh underwent elongation and water uptake, contributing to a well-defined shape. The L/D ratio was the same at all the stages (Figure 3c). When the apples reached a certain size, they entered the transition phase. During the mid-development stages, various physiological changes occurred within the fruit. Chlorophyll production decreased, revealing other pigments that were previously masked. The green color started fading, and other pigments, such as carotenoids and anthocyanins, become more prominent, resulting in a relatively rapid increase in coloration levels. The fruit rapidly transitioned to a red color, which was observed at the mid-development stage, especially at 90–120 DAFB. The coloration in the fruit skin increased rapidly and turned red (Figure 4). As the apples matured, they entered the ripening stage. Color changes are indicative of important chemical changes occurring within the fruit. The breakdown of chlorophyll and the increased accumulation of pigments led to further shifts in coloration. The final coloration patterns of apples results from a combination of pigments, including carotenoids and anthocyanins [42,43]. The stability and balance between these pigments determine the overall coloration and appearance of mature apples. Similar phenomena were also found in the previous studies on "Red Delicious" apple variants [44]. The coloration patterns changed during the developmental stages of fruits and varied among apple variants. Other studies have been conducted on pome fruit and pear, belonging to the Rosaceae family, using similar approaches. Pears were collected at different stages of fruit development to evaluate changes in coloration patterns [32,33]. The results showed that the color development of the fruits changed according to their developmental stage. In general, these changes in fruits varied, and the developmental stages depended on increasing color levels toward the ripening stage.

Additionally, changes in fruit quality were evaluated in addition to fruit enlargement and coloration patterns (Table 1). During fruit development, firmness, TA, and SI decreased, whereas SSC increased. Changes in these fruit quality parameters are part of the natural ripening process of many fruits, including apples, and are driven by complex biochemical and physiological processes in fruits. Toward the ripening stage, firmness declines because of the breakdown of cell walls due to the activity of enzymes, such as pectinases and cellulases [45]. The degradation of these cell components softens the fruit texture [46–48]. TA refers to the amount of acid present in the fruits. During the early stages of fruit development, high acid levels are required to provide a tart or sour taste. However, these acids are metabolized and broken down as the fruit ripens. Enzymes, such as citrate synthase and malate dehydrogenase, play a role in this process. The decline in TA is responsible for the transition from a sour or tangy taste to a sweeter flavor [49]. Starch is stored in the flesh of apples. At the early stages of fruit development, starch is abundant and provides the needed energy to support fruit growth. However, as the apples ripen,

starch is converted into sugar through enzymatic hydrolysis (amylase). These sugars contribute to the increase in SSC and a sweeter taste of the fruit. As starch is broken down into sugars and the water content of the fruit decreases due to evaporation, the concentration of SSC increases [50]. Together, these systematic changes contribute to the sweetness of the fruit, enhance its palatability, and are a key indicator of ripeness. Similar changes were reported in other studies on apples [51] and cherries [52]. In summary, these changes are interconnected and are part of the biochemical processes that occur during fruit development.

4.2. Differential Biosynthesis Gene Expression and Pigment Accumulation According to Fruit Developmental Stages

Transcript expression profiles of anthocyanin and carotenoid biosynthesis-related genes were analyzed at different stages of fruit development in both skin and flesh tissues. The expression levels of the genes in these biosynthetic pathways determined the amount of pigments they encoded. Therefore, the expression of genes involved in the anthocyanin and carotenoid biosynthesis pathways is responsive to the formation of fruit coloration patterns. In general, the expression profile of these genes varied among different developmental stages and between the skin and flesh tissues of the apples (Figures 5b and 6b). As shown in Figure 5b, most anthocyanin biosynthesis-related structural genes were highly expressed in the late stages (150 DAFB and ripe stage) and were observed in both the skin and flesh. However, some genes (*MdCHS*, *MdDFR*, *MdANS*, *MdF3H*, and *MdUFGT*) were highly expressed at the early stages (30 DAFB) but dropped out thereafter, at 60–150 DAFB. The higher expression of anthocyanin biosynthesis-related genes during the early stages of apple development is likely due to a combination of developmental signals and hormonal regulation. During this stage, some genes are highly active in establishing the foundation for future processes. The fruit may focus on establishing the necessary infrastructure for pigmentation, including cell differentiation, and establishing specialized tissues where anthocyanins accumulate. Moreover, in the early stages, the fruit grows fast owing to the cell division and expansion that occur when hormonal concentration is high. Li et al. [53] found that changes in endogenous hormone levels led to changes in total anthocyanin concentrations in the skin of apples during fruit development. Therefore, hormonal signals might promote the activation of anthocyanin-related genes, setting the stage for subsequent coloration. During mid-stage fruit development, the observed decrease in gene expression was likely due to shifting priorities and feedback mechanisms. As the fruit develops, the plant allocates resources and energy differently. Plants in the early stages might prioritize establishing a strong structure and basic metabolic processes, including the synthesis of pigments, such as anthocyanins. As the fruit moves to the middle development stage, energy allocation may shift toward maturation, sugar accumulation, and other processes, leading to a relative decrease in the expression of anthocyanin biosynthesis-related genes. Gene expression is often regulated by feedback loops and complex networks. Once anthocyanin biosynthesis is initiated, feedback mechanisms may down-regulate the expression of related genes to avoid excessive pigment production [54,55]. This may have contributed to the observed decrease in gene expression during the middle stage of fruit development. Similar phenomena were observed in blueberry fruits, with the pattern of anthocyanin biosynthesis genes (*MdDFR* and *MdANS*) being highly expressed at the early and late stages but low at the mid-stage [56].

The transcript expression profiles of the carotenoid biosynthesis genes were also analyzed at different stages of fruit development (Figure 6b). During fruit development, the expression levels of these genes varied across different development stages and between the skin and flesh tissues. The expression of the carotenogenic genes showed an increasing trend. They were expressed at low levels at the early stages but increased thereafter and were highly expressed at the late stages. However, the expression profile of *MdLCYε* did not show an opposite trend. Its expression was high in the early stages and gradually decreased toward the late stages. This could possibly be because in the carotenoid biosynthesis

pathway, the transition from lycopene into β-carotene appeared to be more dominant than transformation into α-carotene. Thus, *MdLCYε*, which encodes the enzyme responsible for catalyzing lycopene into the α-carotene step, might be less expressed; moreover, the focus should be on another direction that requires another encoding gene.

In the present study, the coloration patterns and accumulation of anthocyanins showed a similar trend (Figures 4 and 7). The red color gradually increased during fruit development in relation to the anthocyanin content in both the skin and flesh of the fruit. Pearson's correlation analysis showed a strong correlation between the anthocyanin content and coloration patterns (Table 2). However, there was an intermediate correlation between β-carotene contents and coloration patterns (Table 3), as the coloration and β-carotene contents did not show the same trend (Figures 4 and 8). Furthermore, Pearson's correlation analysis was used to compare coloration patterns and gene expression levels. The expression of anthocyanin biosynthesis-related genes (*MdPAL*, *MdCHI*, and *MdF3H*) was strongly correlated with the coloration patterns. The expression levels of carotenogenic genes (*MdGGPPS*, *MdPSY*, *MdZDS*, *MdCRTISO*, *MdCRHβ*, and *MdZEP*) was strongly to very strongly correlated with coloration patterns.

5. Conclusions

To study the development of coloration in the red-fleshed 'Enbu' apple cultivar, we investigated the changes at different stages of fruit development in the skin and flesh of the fruit. This study showed that as apples developed, coloration increased in a coordinated manner across different developmental stages. Notably, both the skin and flesh tissues showed substantial variations in transcript and pigment concentrations. Through a systematic analysis of the transcript expression of genes related to pigment accumulation during fruit development in correlation with coloration patterns, we identified key transcriptional factors among the genes related to anthocyanin and carotenoid biosynthesis. In addition to pigment content regulation, fruit quality attributes were investigated during fruit development to elucidate the complex relationship between fruit quality and the expression and accumulation of genes involved in pigment biosynthesis that determine color development. These changes are caused by the interplay between various pigments and physiological processes. The accumulation of these pigments is controlled by transcription factors within their respective biosynthesis pathways, and their levels vary due to transcriptional regulation and developmental stages. Understanding the molecular mechanisms involved in pigment biosynthesis at different stages of fruit development, we can apply a range of environmental stimuli such as light and temperature at certain timepoints of fruit development to drive the pigment accumulation. Similar or different phenomena in other apple cultivars or crops might be observed. The differences might be due to different genetic backgrounds; thus, further studies need to be conducted for specific cultivars and crops.

Supplementary Materials: The following supporting information can be downloaded from: https://www.mdpi.com/article/10.3390/horticulturae9101072/s1. Table S1. qRT-PCR primer list used for analysis the expression levels of anthocyanin biosynthesis genes; Table S2. qRT-PCR primer list used for analysis the expression levels of carotenoid biosynthesis genes.

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