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Comparative studies of anthocyanin accumulation and gene expression of flavonoid 3′-hydroxylase during fruit development of two Praratchatan strawberry cultivars

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ABSTRACT: Anthocyanins in strawberry fruit are beneficial to human health. Flavonoid 3′-hydroxylase (F3′H) is a key enzyme in determining the B-ring hydroxylation pattern of flavonoids involved in the anthocyanin production pathway of strawberry fruit. The objectives of this study were to determine the correlation between the types and the amounts of anthocyanins and the expression levels of *F3′H* at four developmental stages of strawberry fruit in two different cultivars: Praratchatan No. 70 and Praratchatan No. 80. The concentration of cyanidin-3-glucoside (cy-3-glc) in the fruit extract samples was analysed using HPLC. Gene expression was determined using RT-qPCR. Colour and pH were measured at each developmental stage. The cy-3-glc concentration was significantly different at each developmental stage ($p < 0.05$), and higher in the Praratchatan No. 70 than in the Praratchatan No. 80. The concentration gradually increased from the early green stage (15 days after anthesis, DAA), to the maximum at the turning stage (25 DAA), and then decreased slightly at the ripe red stage (30 DAA). Gene expression of *F3′H* was positively correlated with the concentration of cy-3-glc at each stage of fruit development. The ripe red stage (25 DAA) was found to be the optimal time to harvest the fruit for healthy consumption.

KEYWORDS: anthocyanin biosynthesis gene, cyanidin-3-glucoside, developmental stages, flavonoid 3′-hydroxylase, strawberry fruit

INTRODUCTION

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) is a natural hybrid of *Fragaria chiloensis* Duch. and *Fragaria virginiana* Duch. It is a perennial, stoloniferous herb belonging to the *Rosaceae* family [1] and is widely grown in all temperate regions of the world. The fruit contains large amounts of flavonoids, including anthocyanins, flavonols, isoflavones, and proanthocyanidins [2].

These flavonoids are antioxidants which can benefit human health, especially anthocyanins, which are among the major flavonoid compounds found in many berries [3, 4]. The two major anthocyanin compounds in strawberry are pelargonidin-3-glucoside (pg-3-glc) (77%–90% of total anthocyanin content) and cyanidin-3-glucoside (cy-3-glc) (3%–10%) [5]. In addition, they play an important role in fruit and vegetable tolerance to environmental stresses and in disease resistance, attracting pollinators, promoting postharvest quality, and extending shelf life [6, 7]. Besides, flavonoids involve in stimulating the immune system [8], protecting against age-related neurological disorders [9], supporting visual functions [10], and preventing inflammation, oxidative stress, cardiovascular disease (CVD), certain types of cancers, type 2 diabetes, obesity, and neurodegeneration [11].

Biotechnology offers methods to capitalize on specific biosynthetic pathways, with the goal of breeding plants with improved nutritional and phytochemical traits [12]. In particular, engineering the flavonoid pathway has proven to be an effective tool for enhancing fruit nutritional value and quality [13]. Therefore, the perspective of consumer health can be further improved by increasing consumption.

However, the types and amounts of anthocyanin are controlled by a group of specific genes and related enzymes through a specific pathway involved in its synthesis [14]. The F3′H is critically involved in anthocyanin biosynthesis. The isoform of F3′H in strawberries has been reported in only one isoform [15]. Various isoforms of F3′H, especially CsF3′H1, CsF3′H2, CsF3′H3, and GbF3′H1, have been successfully used to enhance flavonoid production in the leaves of tea (*Camellia sinensis* L.) and ginkgo (*Ginkgo biloba* L.) [16, 17].

The F3′H enzyme activates the synthesis of cyanidin-derived metabolites and catalyses the introduction of hydroxyl groups into position 3′ of naringenin (NAR), dihydrokaempferol (DHK), kaempferol (KM), and apigenin (API) [15], as well as the conversion of dihydrokaempferol (DHK) to dihydroquercetin (DHQ) [18]. *F3′H* has been studied at the genetic, biochemical, and molecular levels among different strawberry species, including *F.* × *ananassa* cv. Elsanta and *F. vesca* cv. Red Wonder [15]. This gene and the associated enzymes have been shown to change the types and the amounts of anthocyanins during the strawberry fruit development. The anthocyanin content during the immature stages of development

protects the fruit; while at the bright red mature stage, it makes strawberry fruit attractive to consumers. The types and the optimum amounts of anthocyanin during fruit development affect the concentration of specific compounds that indicate the optimal time for harvesting and may also benefit the health of consumers.

Strawberries are widely planted in Northern Thailand. The main cultivars include Praratchatan No. 70 and Praratchatan No. 80. The fruits of these cultivars have conic to globose berry shapes with good aroma, sweetness, redness, and firmness. They also have high nutritional contents, including anti-oxidant compounds [19]. However, there is no research to date on the relationship between anthocyanin production and gene expression of *F3′H* during strawberry fruit development in these cultivars. Knowledge of the relationships between gene expression and the synthesis of anthocyanin compounds has the potential to assist in defining the optimum harvest time for these strawberry cultivars and to provide quantitative information about the types and the amounts of anthocyanins in the fruit during its development. Therefore, the objectives of this study were to determine the correlations between the types and amounts of cy-3-glc produced and the expression levels of *F3′H* in fruit at four developmental stages in the strawberry cultivars Praratchatan No. 70 and Praratchatan No. 80. As F3′H is an important enzyme in determining the B-ring hydroxylation pattern of flavonoids and is also responsible for cy-3-glc production, the information obtained in this study can be helpful in predicting the appropriate time for optimal harvesting of these strawberry cultivars for optimum health benefits.

MATERIALS AND METHODS

Chemicals

All chemical reagents for HPLC analysis, HPLC-grade water, methanol, ethanol, and acetonitrile were used. Anthocyanin standards, including cyanidin 3-glucoside, were purchased from Sigma-Aldrich (Switzerland). For RT-qPCR analysis, RNA isolation kit was from Invitrogen (USA), RedSafe™ from iNtRON Biotechnology (Korea), 100 bp DNA Ladder RTU from Genedirex® (Taiwan), Tetro cDNA Synthesis Kit from Bioline (UK), One PCR™ Plus from Genedirex® (Taiwan), and SensiFastTM SYBR® No-ROX Kit from Bioline (UK).

Plant material and sample collection

Plants were grown in a greenhouse in Wawee Village, Chiang Rai Province in Northern Thailand (latitude: 19.917925, longitude: 99.495306). Thirty-six samples at each developmental stage of the two cultivars, Praratchatan No. 70 and Praratchatan No. 80, were collected at four different developmental stages: early green stage (G, 15 DAA), white stage (W, 20 DAA), turning stage (T, 25 DAA), and ripe red stage (R, 30 DAA) (Fig. 1). After harvest, all fruit samples were frozen in a deep freezer at −80 °C before analysis. Physico-chemical analysis consisted of 30 replications. Gene expression was separately measured on six berries randomly sampled from the 36 berries of each fruit development stage.

Colour and pH analyses of strawberry fruits

The colours of the external fruit surface and the internal flesh were assessed using a colorimeter (Konica Minolta CR-20) and expressed as L^*, a^*, and b^* values along with colour hue angle (h°). The L^* value indicates lightness, a^* is the red/green value, b^* is blue/yellow, and h° is the hue angle expressed in degrees. To determine the pH, a 10 g fruit sample was cut into small pieces and squeezed through a piece of cheesecloth. The pH of the fruit juice obtained was measured using a pH meter (Satorious, Docu pH meter).

Types and amounts of HPLC identified anthocyanins

Extraction of anthocyanins from the fruit samples at the four different developmental stages was conducted according to a previously reported method [20] with slight modifications. Freeze-dried strawberry powder (0.1 g) was added to 5 ml of acidified methanol (0.1% HCl in MeOH). The extracted sample (5 ml) was dried in a centrivap concentrator (Labconco, Kansas City, MO, USA) at 30 °C. The dried sample was then solubilized with 0.1% HCl in MeOH (1000 µl) and filtered through a 0.45 µm VertiPure™ nylon syringe filter prior to HPLC analysis. A 60 µl sample was injected into a Shimadzu analytical HPLC column (Inertsil®ODS-3 5 µm 4.6 × 250 mm in a guard column Inertsil®ODS-3 4.0 × 10 mm). Solutions of (A): acetonitrile + 0.1% formic acid and (B): acetonitrile/water/formic acid (5:94.9:0.1) were used as the mobile phase. The gradient program of solvent A was 0% at zero time and ramped linearly to 20% at 20 min, 30% at 26 min, 50% at 28.5 min, 50% at 28.5 min, 95% at 32 min, and back to 0% at 35 min. The monitoring system was performed using a 520 photodiode array detector (PDA) at a flow rate of 0.8 ml/min with a column temperature of 35 °C. Anthocyanins were quantified and identified using external cy-3-glc calibration curves and calculated as mg/kg fresh weight (FW).

RNA isolation and cDNA synthesis

An approximately 100 mg sample of strawberry fruit at each of the four different developmental stages (G, W, T and R) was ground in a mortar under liquid nitrogen freezing conditions. The ground sample was added to an Eppendorf pipette. For total RNA isolation, reagents from the isolation kit (PureLink® RNA, Invitrogen, USA) were sequentially added to

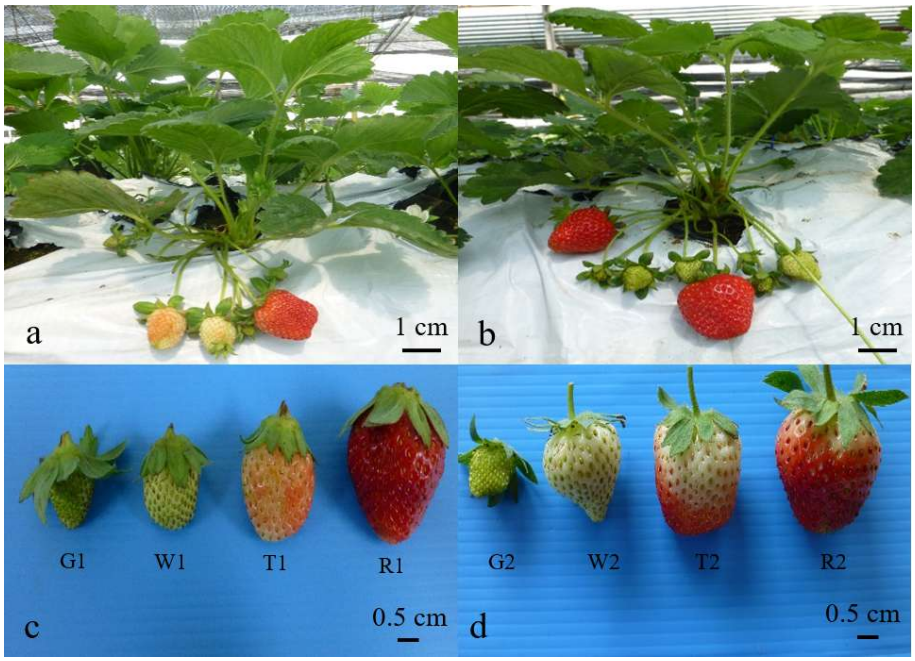

Fig. 1 Morphological characteristics of fruit development in strawberry cultivars. (a) Praratchatan No. 70; (b) Praratchatan No. 80; (c) Praratchatan No. 70; and (d) Praratchatan No. 80 at four developmental stages: G = green stage; W = white stage; T = turning stage; R = ripe red stage.

the sample according to the manufacturer's instructions. The RNA band of the sample was determined on a 1% agarose gel and stained with 1 µl/100 ml RedSafe™ (iNtRON Biotechnology, Korea) in 1X TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0). The appearing bands were visualised and photographed using a SmartView Pro 1200 Image System (Major Science, Taiwan). The individual band intensity was compared with a marker of 100 bp DNA Ladder RTU (Genedirex®, Taiwan). RNA concentration was measured using a microplate reader (Biosan, Latvia). cDNA was synthesised from the isolated RNA using a Tetro cDNA Synthesis Kit (Bioline, UK). The synthesised cDNA was tested using RT-qPCR according to the following procedure.

PCR amplifications optimizing condition test

Primers for *F3′H* and *GAPDH* were designed for RT-qPCR based on previously obtained *F3′H* and *GAPDH* sequences [15, 21]. Two primers were used for PCR amplifications to determine the complementarity of *F3′H* and *GAPDH* (control genes) in the samples from the two cultivars. The PCR-amplified DNA fragments were separated by electrophoresis in submerged horizontal agarose gels (1% w/v) mixed with 1 µl/100 ml RedSafe™ (iNtRON Biotechnology, Korea) in 1X TBE buffer. The resultant bands were visualised and photographed using a SmartView Pro 1200 Image System (Major Science, Taiwan). In the PCR assays, 1 µl of cDNA (1 µg), 1 µl primer forward, reverse (10 µM), one PCR™ Plus (Genedirex®, Taiwan) master mix (10 µl), and DEPC water (7 µl) were mixed in a small Eppendorf pipette for PCR analysis. The PCR reaction was performed as described by Thill et al [15], with slight modifications, and a PCR Thermal Cycler (T100™ Thermal Cycler, Bio-Rad, USA) was used. The PCR reaction consisted of five steps as follows: 1 cycle at 94 °C for 1 min (pre-denaturation), 44 cycles at 94 °C for 30 s (denaturing), 62 °C (*F3′H, GAPDH*) for 1 min (annealing), 72 °C for 1 min (extension), and 72 °C for 10 min (final extension). This optimisation condition for RT-qPCR was selected for the next step of real-time PCR (RT-qPCR).

Gene expression analysis with reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The polymerase chain reactions of the RNA extracted from the fruit samples of the two cultivars were amplified using RT-qPCR under optimised conditions. Gene expression of *F3′H* and *GAPDH* in the four developmental stages was quantified by RT-qPCR using a qTOWER^{3}G Touch (Analytik Jena, Germany) and a SensiFast™ SYBR® No-ROX Kit (Bioline, UK) according to the manufacturer's instructions. The analysis was carried out with six biological replicates, and the data were normalised against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Primers for *F3′H* were designed based on a previously obtained *F3′H* sequence [15]. Each PCR reaction was conducted

according to the method described in the section PCR amplifications optimising condition test. *GAPDH* served as an internal control. Six replicates of each RT-qPCR reaction were performed for each sample. The amplification efficiency of all primers used was determined prior to the sample investigation. Relative expression values were first calculated as $2^{-\Delta\Delta CT}$ [22], which obtained a single product, normalising against the internal control *GAPDH* [21]. The relative transcript abundance value of the fruit at stage G served as a calibrator (1.0) for comparison with other stages [21].

Data analysis and statistics

Data were analysed with SPSS program version 17.0 software for analysis of variance ($p < 0.05$). Significant differences among treatment means were analysed using Duncan's multiple range test (DMRT) at $p < 0.05$. Results are expressed as mean ± standard deviation.

RESULTS

Colour and pH analyses

The L*, b*, and h° values for both the external fruit surface and the internal flesh at each of the four different stages of Praratchatan No. 70 were typically lower, and a* values were higher than those of Praratchatan No. 80. In addition, the pH values of the Praratchatan No. 70 were consistently lower than those of the Praratchatan No. 80 (Table 1).

Types and amounts of cy-3-glc determination

The results showed that the Praratchatan No. 70 produced a three-fold higher amount of cy-3-glc than the Praratchatan No. 80 (16.78 mg/kg FW at 25 DAA at each of the four development stages). The concentration was the lowest at the G stage (the green stage), gradually increased to the maximum level at the T stage (turning stage), and then slightly decreased at R stage (ripe red stage). The amounts of anthocyanins at each of the four stages were, respectively, 1.30, 5.31, 16.78, and 11.04 mg/kg FW in Praratchatan No. 70 and 1.10, 2.50, 4.79, and 3.89 mg/kg FW in Praratchatan No. 80 (Fig. 2).

Gene expression of *F3′H*

F3′H controls anthocyanin production in strawberry fruit. The gene expression level of *F3′H* at four different developmental stages was higher in the Praratchatan No. 70 than in the Praratchatan No. 80 (Fig. 3). The expression level of *F3′H* increased to a maximum at the T stage (25 DAA), and then gradually decreased to almost no activity at the R stage (30 DAA).

DISCUSSION

Anthocyanins are widely studied for their beneficial effects on health, both in human and animal models [3, 13, 23, 24]. Various fleshy fruits, including strawberry, are a major source of anthocyanins in the human diet [6, 7]. Fruit colour is one of the most important quality criteria for strawberries with respect to purchasing behaviour and consumption [25, 26]. The colour of strawberry fruit typically depends on both the a* and the h° values [27]. In this study (Table 1),

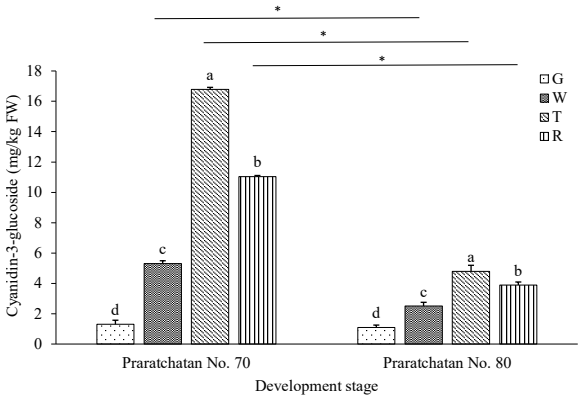

Fig. 2 Changes in amounts of cy-3-glc in two strawberry cultivars, Praratchatan No. 70 and Praratchatan No. 80, at four different developmental stages: G, green stage (15 DAA); W, white stage (20 DAA); T, turning stage (25 DAA); and R, ripe red stage (30 DAA). DAA = day after anthesis. Differences between small letters within the same cultivar indicate a significant difference between means of strawberry cultivars at $p < 0.05$. Asterisk (*) indicates a significant ($p < 0.05$) difference between the same developmental stages of Praratchatan No. 70 and Praratchatan No. 80.

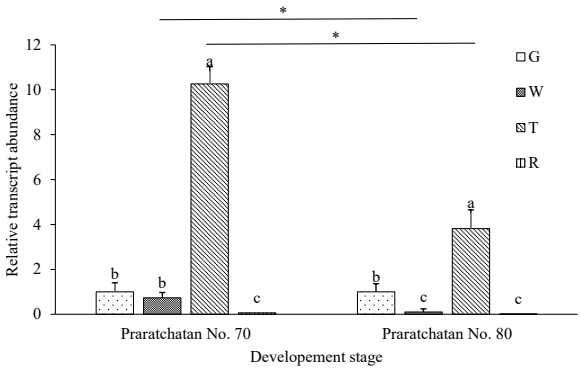

Fig. 3 Quantitative expression of the *F3′H* controlling anthocyanin production two strawberry cultivars, Praratchatan No. 70 and Praratchatan No. 80, at four different developmental stages: G, green stage (control); W, white stage; T, turning stage; and R, ripe red stage. Differences between small letters within the same cultivar indicate a significant difference between the means of strawberry cultivars at $p < 0.05$. Asterisk (*) indicates a significant ($p < 0.05$) difference between the same developmental stages of Praratchatan No. 70 and Praratchatan No. 80.

Table 1 External fruit surface and the internal flesh colour and pH of the two strawberry cultivars, Praratchatan No. 70 and Praratchatan No. 80, at different developmental stages.

Colour/pH		Praratchatan No. 70				Praratchatan No. 80			
		G†	W	T	R	G†	W	T	R
Surface	L*	$50.26 \pm 3.40^{b*}$	$57.33 \pm 2.70^{a*}$	$32.56 \pm 2.95^{c*}$	$29.60 \pm 2.04^{c*}$	59.46 ± 1.87^{B}	62.13 ± 0.81^{A}	40.60 ± 1.05^{C}	38.96 ± 1.02^{C}
	a*	$-1.50 \pm 0.98^{d*}$	$3.00 \pm 0.62^{c*}$	$32.13 \pm 1.50^{a*}$	$28.13 \pm 1.44^{b*}$	-2.90 ± 0.20^{C}	1.83 ± 0.30^{B}	24.73 ± 0.55^{A}	25.00 ± 0.62^{A}
	b*	$27.66 \pm 2.47^{a*}$	$21.03 \pm 2.49^{b*}$	$18.36 \pm 1.96^{b*}$	$12.66 \pm 1.84^{c*}$	32.13 ± 2.21^{A}	28.53 ± 1.15^{B}	20.63 ± 0.61^{C}	17.40 ± 0.55^{D}
	h°	$93.03 \pm 1.93^{a*}$	$81.90 \pm 0.65^{b*}$	$29.63 \pm 1.91^{c*}$	$24.10 \pm 2.10^{d*}$	94.65 ± 1.95^{A}	84.41 ± 2.45^{B}	32.70 ± 1.11^{C}	27.40 ± 0.78^{D}
Flesh	L*	$68.43 \pm 0.23^{a*}$	$68.36 \pm 1.70^{a*}$	$61.53 \pm 1.12^{b*}$	$57.00 \pm 2.66^{c*}$	74.43 ± 1.17^{A}	71.83 ± 0.45^{B}	71.86 ± 0.20^{B}	68.86 ± 0.56^{C}
	a*	$-1.40 \pm 0.17^{c*}$	$-0.53 \pm 0.20^{c*}$	$16.83 \pm 1.56^{b*}$	$21.83 \pm 3.26^{a*}$	-2.33 ± 0.25^{C}	-1.16 ± 0.15^{B}	13.23 ± 0.85^{A}	13.80 ± 0.65^{A}
	b*	$22.26 \pm 0.58^{a*}$	$13.43 \pm 1.05^{c*}$	$14.30 \pm 0.85^{bc*}$	$15.90 \pm 1.70^{b*}$	29.03 ± 1.09^{A}	17.96 ± 0.47^{C}	20.70 ± 1.40^{B}	17.30 ± 0.78^{C}
	h°	$93.56 \pm 0.37^{a*}$	$90.23 \pm 2.90^{b*}$	$40.40 \pm 1.21^{c*}$	$36.16 \pm 1.20^{d*}$	94.25 ± 1.62^{A}	91.57 ± 1.65^{B}	42.19 ± 0.91^{C}	38.63 ± 0.75^{D}
pH		$4.29 \pm 0.20^{a*}$	$2.53 \pm 0.52^{d*}$	$2.59 \pm 0.28^{c*}$	$2.76 \pm 0.32^{b*}$	4.43 ± 0.66^{A}	2.68 ± 0.30^{D}	2.78 ± 0.20^{C}	2.90 ± 0.45^{B}

† Mean values (±SD); n = 30; means with different lowercase and uppercase letters within the same row are significantly different between development stages within each strawberry cultivar ($p < 0.05$).

* Asterisk indicates a significant difference between the developmental stages of Praratchatan No. 70 and Praratchatan No. 80 ($p < 0.05$). G, green stage; W, white stage; T, turning stage; R, ripe red stage.

the lowest h° of fruit samples was found at 30 DAA, indicating a darker red coloration than that at the other developmental stages. However, when considering the relationship between anthocyanin concentration and consumption, the results clearly showed that the turning stage at 25 DAA had the highest concentration of anthocyanins (Fig. 2). Therefore, harvesting for consumption might be better at a slightly less mature stage (at 25 DAA instead of 30 DAA) if health benefits are the main objective. The anthocyanin (cy-3-glc) concentration determines the red colour of strawberries [27]. The b* values are indicative of the yellow pigments in the external surface and the internal flesh of the fruit, which are mainly dependent on β-carotene (pro-vitamin A) concentration [28]. The b* value was associated with the h° value; the higher the b* value, the higher the h° value. The yellow pigments are primarily flavonoids, which include phytoene, z-carotene, lycopene, α-carotene, lutein, β-carotene, β-cryptoxanthin, zeaxanthin, all-trans-violaxanthin, and 3-cis-violaxanthin. These pigments are found in a variety of fruit species, but only β-carotene is found in the strawberry cultivar Akihime [28]. The high h° value found in Praratchatan No. 80 might indicate that there was high concentration of β-carotene in the external surface of the fruit. Hence, a lower h° value was observed in the external surface of the Praratchatan No. 70, indicating a lower β-carotene concentration. Previous findings have shown that the h° value correlates strongly with β-carotene ($r = 0.99$) [29]. β-carotene is a pro-vitamin A, which is beneficial to human health. However, no study has been conducted on the amount of β-carotene in the Praratchatan No. 70 and Praratchatan No. 80. The b* values in both the external surface and the internal flesh tended to be similar between the two cultivars.

As in other studies, the anthocyanin concentration in the external surface was found to be higher than that in the internal flesh of the fruit [30, 31]. Previous findings have shown a high correlation between cy-3-glc content and a* values in the cvs. Elsanta and Miss, similar to the results obtained in this study [27].

pH is an important factor which can affect the colour of different anthocyanin compounds [32, 33]. The Praratchatan No. 70 had a lower pH than that of the Praratchatan No. 80 (Table 1). The concentration of cy-3-glc found in Praratchatan No. 70 was higher than the Praratchatan No. 80; therefore, the former had a darker ripe red colour than the later. A previous study has showed that cyanidin becomes redder when the pH is below 3, appears violet at pH 7–8, and turns blue at very high pH values (pH > 11) [34]. These results are similar to those obtained with anthocyadinins at a low pH (acidic conditions); and anthocyadinins, such as cyanidin, are highly soluble in water due to the formation of the flavylium cation which has a red appearance [35]. Another study has shown that with a change in pH from 4 to below 3 in the early stages of fruit development, there is little change from green to red due to the domination of the accumulated pro-anthocyanin during those stages of development [36]. In the later developmental stage, from the G stage (15 DAA) to the T stage (25 DAA), when the pH was below 3 and there was accumulation of cy-3-glc (anthocyanin), the colour changed to red (the a* value increased).

Our results showed that the cy-3-glc values in the fruit samples were the highest at the T stage (25 DAA) and then decreased slightly at the R stage (30 DAA) in the two strawberry cultivars studied. These results were consistent with the low anthocyanin concentration at the G stage (15 DAA), while the pro-anthocyanin accumulation was the highest at this stage; whereas at the T stage (25 DAA), the anthocyanin concentration was high, and the pro-anthocyanin was at a minimum [35]. In addition, the

anthocyanin content (cy-3-glc) depends on many factors including genotype, harvest period, and climatic conditions [5, 37, 38].

The cy-3-glc concentrations in the strawberry cultivar Praratchatan No. 70 produced approximately 16.78 mg/kg FW, which was a little higher than the concentrations found in other cultivars; e.g., Carisma and Oso Grande (13.0 and 11.0 mg/kg FW, respectively) [5]. Moreover, the concentration of cy-3-glc in the strawberry cultivar Praratchatan No. 70 was higher than that in the three new breeding lines: BL 2005-180 (10.6 mg/kg FW), BL 2005-188 (12.7 mg/kg FW), BL 2006-221-22 (15.7 mg/kg FW), and Joy (12.8 mg/kg FW); Rubygem (9.5 mg/kg FW); and Sugarbaby (5.1 mg/kg FW) a commercial strawberry cultivar [39]. Besides, the concentrations of cy-3-glc in several other strawberry cultivars have been reported in the range of 4.2–14.0 mg/kg FW [40]. Our result indicates that Praratchatan No. 70 has a high potential for commercial production as a health-promoting product.

The expression level of *F3′H* was the highest at the T stage (25 DAA) compared with the control (G stage) ($p < 0.05$). Previous studies have shown that the *F3′H* transcript levels in *F. vesca* cv. Red Wonder increased during fruit ripening with a transcription peak in stage 3 (late turning stage), whereas *F3′H* expression in *F.* × *ananassa* cv. Elsanta decreases during fruit development [15]. However, different results might be caused not only by species but also by different environments [36]. During the early developmental stages, pro-anthocyanidin is the predominant pigment, and the accumulation of this pigment is associated with the upregulation of *F3′H* transcript levels, but caused downregulation at thelater stages [40]. In addition, *F3′H* expression related to the production of anthocyanins and flavonoids was transcribed at the early developmental stages of strawberry fruits [15]. Thus, *F3′H* is the key gene that controls the production of both the B-ring dihydroxylated flavonoids in the unripe strawberry fruits and monohydroxylated flavonoids in the ripe fruits. Consequently, *F3′H* directs the optimisation of flavonoid composition at different physiological stages of *F.* × *ananassa*. The role of *F3′H* is related to the synthesis of a key enzyme at the branch point in the pathway of anthocyanin production, such as the conversion of dihydrokaempferol (DHK) to dihydroquercetin (DHQ), leading to the synthesis of cyanidin derivatives [15, 18].

CONCLUSION

The results from the present study show that the expression patterns of *F3′H* are consistent with the production of anthocyanins. *F3′H* was highly expressed in the T stage (25 DAA), and its expression patterns were correlated with the amount of anthocyanins (cyanidin-3-glucoside) during the fruit development. These results indicate that 25 DAA is an appropriate time to harvest the fruit for consumption, as this is the stage at which the highest concentration of cy-3-glc is produced.

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REFERENCES

1. Sakila S, Ahmed MB, Roy UK, Biswas MK, Karim R, Razvy MA, Hossain M, Islam R, et al (2007) Micropropagation of strawberry (*Fragaria* × *ananassa* Duch.) a newly introduced crop in Bangladesh. *Am-Euras J Sci Res* **2**, 151–154.
2. Dixon RA, Sumner LW (2003) Legume natural products: Understanding and manipulating complex pathways for human and animal health. *Plant Physiol* **131**, 878–885.
3. Capocasa F, Scalzo J, Mezzetti B, Battino M (2008) Combining quality and antioxidant attributes in the strawberry: The role of genotype. *Food Chem* **111**, 872–878.
4. Peña-Sanhueza D, Inostroza-Blancheteau C, Ribera A, Reyes-Díaz M (2017) Anthocyanins in berries and their potential use in human health. In: Shiomi N (ed) *Superfood and Functional Food – The Development of Superfoods and Their Roles as Medicine*, Intech Open Limited, United Kingdom, pp 155–172.
5. Lopes-da-Silva F, Escribano-Bailòn MT, Pérez Alonso JJ, Rivas-Gonzalo JC, Santos-Buelga C (2007) Anthocyanin pigments in strawberry. *LWT Food Sci Technol* **40**, 374–382.
6. Petroni K, Tonelli C (2011) Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Sci* **181**, 219–229.
7. Jaakola L (2013) New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends Plant Sci* **18**, 477–483.
8. Fu Y, Yu JC, Ding XR, Han J (2009) Effects of acupuncture on expressions of the transcription factors NF-E2, YB-1, LRG47 in the SAMP10 mice. *J Tradit Chin Med* **29**, 54–59.
9. Shin WH, Park SJ, Kim EJ (2006) Protective effect of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats. *Life Sci* **79**, 130–137.
10. Hope Smith S, Tate PL, Huang G, Magee JB, Meepagala KM, Wedge DE, Larcom LL (2004) Antimutagenic activity of berry extracts. *J Med Food* **7**, 450–455.
11. Giampieri F, Tulipani S, Alvarez-Suarez JM, Quiles JL, Mezzetti B, Battino M (2012) The strawberry: Composition, nutritional quality, and impact on human health. *Nutrition* **28**, 9–19.

12. Diamanti J, Capocasa F, Balducci F, Battino M, Hancock J, Mezzetti B (2012) Increasing strawberry fruit sensorial and nutritional quality using wild and cultivated germplasm. *PLoS One* **7**, e0046470.
13. Giampieri F, Gasparrini M, Forbes-Hernandez TY, Mazzoni L, Capocasa F, Sabbadini S, Alvarez-Suarez JM, Afrin S, et al (2018) Overexpression of the anthocyanidin synthase gene in strawberry enhances antioxidant capacity and cytotoxic effects on human hepatic cancer cells. *J Agric Food Chem* **66**, 581–592.
14. Provenzano S, Spelt C, Hosokawa S, Nakamura N, Brugliera F, Demelis L, Geerke DP, Schubert A, et al (2014) Genetic control and evolution of anthocyanin methylation. *Plant Physiol* **165**, 962–977.
15. Thill J, Miosic S, Gotame TP, Mikulic-Petkovsek M, Gosch C, Veberic R, Preuss A, Schwab W, et al (2013) Differential expression of flavonoid 3′ hydroxylase during fruit development establishes the different B-ring hydroxylation patterns of flavonoids in *Fragaria* × *ananassa* and *Fragaria vesca*. *Plant Physiol Biochem* **72**, 72–78.
16. Wei K, Wang L, Zhang C, Wu L, Li H, Zhang F, Cheng H (2015) Transcriptome analysis reveals key flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase genes in affecting the ratio of dihydroxylated to trihydroxylated catechins in *Camellia sinensis*. *PLoS One* **10**, e0137925.
17. Wu Y, Wang T, Xin Y, Wang G, Xu L-A (2020) Overexpression of the *GbF3′H1* gene enhanced the epigallocatechin, gallocatechin, and catechin contents in transgenic *populus*. *J Agric Food Chem* **68**, 998–1006.
18. Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* **7**, 1071–1083.
19. Sirijan M, Pipattanawong N, Chaiprasart P (2019) Effect of 1-naphthalene acetic acid and 6-benzyladenine on micropropagation of strawberry cultivar 'Praratchatan No. 80'. *Agric Nat Resour* **53**, 355–363.
20. Palapol Y, Ketsa S, Stevenson D, Cooney JM, Allan AC, Ferguson, IB (2009) Colour development and quality of mangosteen (*Garcinia mangostana* L.) fruit during ripening and after harvest. *Postharvest Biol Technol* **51**, 349–353.
21. Salvatierra A, Pimentel P, Moya-Leon MA, Caligari PD, Herrera R (2010) Comparison of transcriptional profiles of flavonoid genes and anthocyanin contents during fruit development of two botanical forms of *Fragaria chiloensis* ssp. chiloensis. *Phytochem* **71**, 1839–1847.
22. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3**, 1101–1108.
23. He J, Giusti MM (2010) Anthocyanins: natural colorants with health-promoting properties. *Annu Rev Food Sci Technol* **1**, 163–187.
24. Nas JSB, Manalo RVM, Medina PMB (2021) Peonidin-3-glucoside extends the lifespan of *Caenorhabditis elegans* and enhances its tolerance to heat, UV, and oxidative stresses. *ScienceAsia* **47**, 457–468.
25. Hoffmann T, Kalinowski G, Schwab W (2006) RNAi-induced silencing of gene expression in strawberry fruit (*Fragaria* × *ananassa*) by agroinfiltration: a rapid assay for gene function analysis. *Plant J* **48**, 818–826.
26. Schlangen K, Miosic S, Halbwirth H (2010) Allelic variants from *Dahlia variabilis* encode flavonoid 3′-hydroxylases with functional differences in chalcone 3-hydroxylase activity. *Arch Biochem Biophys* **494**, 40–45.
27. Voča S, Žlabur JŠ, Dobričevič N, Jakobek L, Šeruga M, Galič A, Pliestič S (2014) Variation in the bioactive compound content at three ripening stages of strawberry fruit. *Molecules* **19**, 10370–10385.
28. Yano M, Kato M, Ikoma Y, Kawasaki A, Fukazawa Y, Sugiura M, Matsumoto H, Oohara Y, et al (2005) Quantitation of carotenoids in raw and processed fruits in Japan. *Food Sci Technol Res* **11**, 13–18.
29. Simonne AH, Kays SJ, Koehler PE, Eitenmiller RR (1993) Assessment of beta-carotene content in sweet potato (*Ipomoea batatas* Lam.) breeding lines in relation to dietary requirements. *J Food Compos Anal* **6**, 336–345.
30. Giomaro G, Karioti A, Bilia AR, Bucchini A, Giamperi L, Ricci D, Fraternale D (2014) Polyphenols profile and antioxidant activity of skin and pulp of a rare apple from Marche region (Italy). *Chem Cent J* **8**, 45.
31. Yin L, Chen T, Li Y, Fu S, Li L, Xu M, Niu Y (2016) A comparative study on total anthocyanin content, composition of anthocyanidin, total phenolic content and antioxidant activity of pigmented potato external surface and flesh. *Food Sci Technol Res* **22**, 219–226.
32. Turturică M, Oancea AM, Râpeanu G, Bahrim G (2015) Anthocyanins: naturally occurring fruit pigments with functional properties. *AUDJG Food Technology* **39**, 9–24.
33. Khoo HE, Azlan A, Tang ST, Lim SM (2017) Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food Nutr Res* **61**, ID 1257.
34. Torskangerpoll K, Andersen M (2005) Colour stability of anthocyanins in aqueous solutions at various pH values. *Food Chem* **89**, 427–440.
35. Khoo HE, Chew LY, Ismail A, Azlan A (2012) Anthocyanins in purple-colored fruits. In: Sun J, Prasad KN, Ismail A, Yang B, You X, Li L (eds) *Polyphenols: Chemistry, Dietary Sources and Health Benefits*, Nova Science Publisher, New York, pp 133–152.
36. Zhang X, Chen Q, Yu D, Zhou S, Tang H (2013) Expression analysis of the ANR and LAR gene in *Fragaria* × *ananassa* cv. Toyonaka. *J Agric Sci* **5**, 64–69.
37. Stapleton SC, Chandler CK, Legard DE, Price JF, Jr Sumler JC (2001) Transplant source affects fruiting performance and pests of 'sweet Charlie' strawberry in Florida. *HortTechnol* **11**, 61–65.
38. Bakowska A, Kucharska AZ, Oszmiański, J (2003) The effects of heating, UV irradiation, and storage on stability of the anthocyanin-polyphenol copigment complex. *Food Chem* **81**, 349–355.
39. Fredericks CH, Fanning KJ, Gidley MJ, Netzel G, Zabaras D, Herrington M, Netzel M (2013) High-anthocyanin strawberries through cultivar selection. *J Sci Food Agric* **93**, 846–852.
40. Donno D, Cavanna M, Beccaro GL, Mellano MG, Marinoni DT, Cerutti AK, Bounous G (2013) Currants and strawberries as bioactive compound sources: determination of antioxidant profiles with HPLC-DAD/MS. *J Appl Bot Food Qual* **86**, 1–10.