Anthocyanin Production and Enzymatic Degradation during the Development of Dark Purple and Lilac Paprika Fruit

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ABSTRACT. During development, the fruit of some paprika (*Capsicum annuum* L.) cultivars shows a change in color from green to dark purple (e.g., ‘Mavras’) or lilac (e.g., ‘Tequila’). However, this purple coloration is rare among paprika cultivars and disappears in ripened fruit, which are red. Therefore, we investigated the mechanism causing this color change in the cultivars Mavras and Tequila to better understand how purple ripened fruit could be generated. High-performance liquid chromatography (HPLC) analyses of the anthocyanin contents of the fruit indicated that anthocyanin was undetectable in green fruit, accumulated in dark purple or lilac ones, and then decreased again in red ones in both cultivars. Furthermore, expressions of most of the analyzed anthocyanin biosynthesis-related genes and genes for their transcription factors increased in dark purple or lilac fruit and decreased in red ones, i.e., it was synchronized with the changes in anthocyanin contents. Furthermore, anthocyanin degradation activity as a result of peroxidases was detected at all stages but increased when the lilac or dark purple color started to fade. Thus, the development of purple coloration is caused by increased anthocyanin biosynthesis, whereas the fading of this coloration is a result of both a decrease in anthocyanin biosynthesis and an increase in anthocyanin degradation. At the ripening stage, the green pigment (chlorophyll) contents decreased, whereas the red pigment (carotenoid, particularly capsanthin) contents increased. However, these timings did not completely coincide with the timing of anthocyanin degradation, suggesting that the content of each pigment is individually regulated, and so purple, green, and red coloration could be freely expressed in mature paprika fruit.

Color is one of the most important indicators of fruit quality for consumers and it also decides the market value. Different cultivars of paprika produce different colored fruit, with eight colors being recognized: red, yellow, orange, green, white, brown, purple, and black (Simonne et al., 1997). Among these, purple fruit have particularly good market potential because purple vegetables are rare. However, although purple cultivars change color from green to purple during development, they then become red at maturity (Fig. 1), making the commercial use of purple paprika problematic.

The purple anthocyanin nasunin (delphinidin-3-p-coumaroyl-rutinoside-5-glucoside) was isolated from eggplant (*Solanum melongena* L.) peels by Kuroda and Wada (1933). It is also one of the major pigments responsible for the purple coloration of the paprika fruit (Aza-González and Ochoa-Alejo, 2012; Lightbourn

et al., 2008). Thus, the change in purple color intensity that occurs in paprika fruit during development appears to be caused by changes in the anthocyanin contents, suggesting that both anthocyanin biosynthesis and degradation are involved in this process.

Anthocyanin biosynthetic activity can be estimated by measuring the expression levels of anthocyanin biosynthesis-related genes, which are regulated by a complex of transcription factors that are encoded by the *MYB*, basic helix–loop–helix (*bHLH*), and WD40-repeat (*WDR*) genes (Koes et al., 2005; Ramsay and Glover, 2005; Xu et al., 2015). In paprika, the expressions of some anthocyanin biosynthesis-related genes are upregulated by *MYB* and *MYC/bHLH* (Aguilar-Barragán and Ochoa-Alejo, 2014; Lu et al., 2018; Zhang et al., 2015), and unripe fruit have higher expressions than ripe ones of *MYB* in the purple inbred line 5226 (Borovsky et al., 2004) and of anthocyanin biosynthesis-related genes in the purple cultivars Arvol and Uvilla (Aza-González et al., 2013). The enzymatic degradation of anthocyanin has also been detected in plants

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Fig. 1. Fruit of the paprika cultivars Mavras and Tequila at the indicated number of days after pollination.

(Barbagallo et al., 2007; Fang et al., 2015; Vaknin et al., 2005; Zipor et al., 2015), which may involve peroxidases, polyphenol oxidases, and β -glucosidases (reviewed by Oren-Shamir, 2009).

Based on this information, we investigated the mechanism behind the change in purple coloration in the paprika fruit by examining changes in the anthocyanin biosynthesis and degradation activities in the fruit of the cultivar Mavras, which are dark purple when immature, and Tequila, which are lilac when immature. We also studied the synchrony between content changes in anthocyanins and both chlorophylls (green pigments) and carotenoids (red pigments) during fruit development to determine whether they are regulated by the same mechanism.

Materials and Methods

PLANT MATERIALS. The paprika cultivars Mavras and Tequila were obtained from Enza Zaden (Enkhuizen, The Netherlands) and a Japanese distributor, respectively. Seeds of each cultivar were sown on a 72-cell plug tray (300 $\times$ 600 $\times$ 50 mm) filled with commercial medium (Yosaku N-150; ZEN-NOU, Tokyo, Japan) on 28 Feb. (Expt. 1) and 1 Sept. (Expt. 2) 2017. After 2 weeks, the seedlings were transplanted into containers (600 $\times$ 400 $\times$ 200 mm) at a density of two seedlings per container and grown hydroponically (EC, 1.2–1.5 mS $\cdot$ cm $^{-1}$; pH 5.6–6.3) in a mixture of two commercial nutrient solutions (OAT House 1 gou and OAT House 2 gou; OAT Agrio, Osaka, Japan) in a glasshouse at the Ikuta campus of Meiji University (Kawasaki, Japan). To avoid exposure to extreme temperatures, the roof and side windows of the glasshouse were opened when the temperature inside the glasshouse reached 25 $^{\circ}$ C, the ceiling fan was activated at 30 $^{\circ}$ C, and a heater was operated when the temperature fell below 12 $^{\circ}$ C. No other environmental conditions (e.g., relative humidity and light) were measured inside the glasshouse.

Eight plants per cultivar were used in each experiment. Pollination was performed by gently shaking the flowers by hand, and fruit were harvested at 10, 15, 20, 40, 50, and 60 d after pollination (DAP) for each cultivar (Fig. 1). We sampled 1–2 g of pericarp from individual different fruit and flash-frozen in liquid nitrogen and stored at -80° C.

QUANTITATIVE ANALYSES OF ANTHOCYANIN, CINNAMIC ACID DERIVATIVE, AND FLAVONOID CONTENTS. The anthocyanin, cinnamic acid derivative, and flavonoid contents of the fruit were measured following Ikeda et al. (2011) with some modifica-

tions. Samples were ground with a mortar and pestle, extracted in six times its volume of 10% (v/v) acetic acid overnight at 4 $^{\circ}$ C, and centrifuged at 12,000 g_n for 3 min (CF15RXII; Hitachi, Tokyo, Japan). The supernatant (10 μ L) was then analyzed on a HPLC system with photodiode array detector (EZChrom Elite; Hitachi) and generic description 4.6 $\times$ 250-mm column (Inertsil ODS-2; GL Science, Tokyo, Japan). HPLC separation was performed at 40 $^{\circ}$ C with a flow rate of 0.8 mL $\cdot$ min $^{-1}$ and a linear gradient of 20% to 100% solvent B (1.5% phosphoric acid, 20% acetic acid, and 25% acetonitrile) in solvent A (1.5% phosphoric acid) over 40 min. Absorption spectra were monitored at 220–600 nm and anthocyanins, cinnamic acid derivatives, and flavonoids were distinguished based on their absorption maxima (525, 330, and 360 nm, respectively). The absorption at each of these wavelengths was used to quantify the amount of each component as an equivalent value of delphinidin glucoside, caffeic acid, and rutin (all from Funakoshi Co., Tokyo, Japan), respectively.

IDENTIFICATION OF ANTHOCYANINS. Anthocyanins were extracted with 10% (v/v) acetic acid from the pericarps of fruit of ‘Mavras’ and ‘Tequila’ at 20 DAP as well as from those of a commercially purchased eggplant of Japanese cultivar. Both the paprika and eggplant extracts were loaded on thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and the plates were developed with 10% acetic acid or n-butanol/acetic acid/distilled water at a 4:1:2 (v/v/v) ratio for 70 mm from sample spot.

‘Tequila’ and the Japanese eggplant extracts were analyzed by HPLC (HP1100; Agilent Technologies, Santa Clara, CA) using the same conditions in the EZChrom Elite HPLC system to compare retention time and absorption spectrum of major anthocyanin in each extract. Separately, ‘Tequila’ and ‘Mavras’ extracts were analyzed by the EZChrom Elite HPLC system to compare retention time of a major anthocyanin in each extract.

QUANTITATIVE ANALYSES OF CHLOROPHYLL AND CAROTENOID CONTENTS. The chlorophyll and carotenoid contents of the fruit were measured as described by Kishimoto et al. (2007) with some modifications. Samples from individual different fruit were ground in liquid nitrogen with a mortar and pestle, mixed with acetone/diethyl ether/Tris-HCl buffer (pH 8.0) [10:30:1 (v/v/v)], and centrifuged at 12,000 g_n for 3 min. Separate aliquots of the supernatants were then used to analyze the chlorophyll and carotenoid contents. For the carotenoid analysis, the supernatant was first saponified with 10% methanolic KOH for 1 h at 4 $^{\circ}$ C in the dark. For both analyses, the supernatant was then evaporated at 37 $^{\circ}$ C in a rotary evaporator (N-1000; Tokyo Rikakikai, Tokyo, Japan) with a water bath (SB-1000; Tokyo Rikakikai), the residue was dissolved in acetone, and the pigments were separated by the EZChrom Elite HPLC on YMC column (Carotenoid S-5 μ m, 250 $\times$ 4.6 mm; Separation Technology, Sanford, FL). The HPLC analysis conditions for both the chlorophylls and carotenoids were as described in Kishimoto et al. (2007). Pigments were distinguished on the basis of the absorption spectra, with maxima of around 440 nm for chlorophylls and 480 nm for carotenoids. The absorption at each of these wavelengths was used to quantify the amount of each component. Chlorophylls and carotenoids in paprika fruit were quantified by drawing a standard curve with the standard.

IDENTIFICATION OF CHLOROPHYLLS AND CAROTENOIDS. Standards of chlorophyll *a*, chlorophyll *b*, β -carotene, lutein,

zeaxanthin, violaxanthin, capsanthin, and capsorubin were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). These standard samples and the extracted compounds were analyzed by HPLC under the same conditions as described previously, and the retention times and absorption spectra (400–510 nm) were compared.

RNA EXTRACTION AND QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR). Total RNA extraction was performed following the methods of Matsushita et al. (2016) and Okutsu et al. (2018), which were based on the procedure of Reid et al. (2006). Total RNA was extracted in 1 M Tris–HCl buffer (pH 8.0) containing 0.5 M ethylenediaminetetraacetic acid (EDTA), 5 M NaCl, 2% cetyltrimethylammonium bromide (Merck), 0.05% spermidine trihydrochloride (Merck), and 2% polyvinylpyrrolidone (PVPP) (Merck). RNA concentration was measured with a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA). cDNA was then synthesized from the isolated RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time; Takara Bio, Shiga, Japan) and was used for qPCR.

The expressions of the following genes were measured by qPCR: the anthocyanin biosynthesis-related genes phenylalanine ammonia-lyase [*PAL* (DQ229940)], chalcone synthase [*CHS* (FJ705842)], chalcone isomerase [*CHI* (FJ705843)], flavanone 3-hydroxylase [*F3H* (FJ705844)], flavonoid 3′5′-hydroxylase [*F3′5′H* (FJ705845)], dihydroflavonol 4-reductase [*DFR*; (FJ705846)], anthocyanidin synthase [*ANS*; (FJ705847)], and UDP glucose: flavonoid 3-O-glucosyltransferase [*UGT*; (FJ705848)]; and the transcription factor genes *MYB* (AJ608992), *bHLH* (no accession number), and *WDR* (no accession number). Specific primer sets for *PAL*, *F3′5′H*, *ANS*, *MYB*, *bHLH*, and *WDR* were adopted from Aza-González et al. (2013) and Zhang et al. (2015), whereas primer sequences for the other genes were based on sequences contained in the GenBank database and were designed with the Primer-BLAST software (National Center for Biotechnology Information, 2019). The primer sequences are listed in Supplemental Table 1. qPCR was performed in an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific) using a PCR protocol of one cycle of denaturation at 95 °C for 30 s, followed by 50 cycles of denaturation at 94 °C for 15 s and annealing/extension at 60 °C for 60 s. Because Zhang et al. (2015) used ubiquitin as a reference gene, ubiquitin [*UBI* (no accession number)] was used as a reference gene for calculating relative expression based on the comparative CT method described by Schmittgen and Livak (2008). The following equation was used to investigate relative expression of the target genes with respect to the reference gene:

$$\Delta Ct = Ct \text{ (target)} - Ct \text{ (reference)},$$

$$\text{Relative value} = 2^{-\Delta Ct}.$$

qPCR was run for three technical replicates per sample.

ANTHOCYANIN-DEGRADING ENZYME EXTRACTION AND ANTHOCYANIN DEGRADATION ASSAY. Samples were ground in liquid nitrogen with a mortar and pestle and extracted in 10 mL of enzyme extraction solution modified from that used by Zipor et al. (2015) [20 mM sodium acetate (pH 5), 20 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (Fujifilm Wako Pure Chemical), and 2.5% PVPP (Merck)]. The homogenate was centrifuged at

12,000 g_n for 10 min at 4 °C. The protein concentration in the supernatant was then measured by the Lowry assay (Bio-Rad, Hercules, CA) with a spectrophotometer [NanoDrop 2000 (650 nm)] using bovine serum albumin as a standard and was considered to represent the protein concentration of crude anthocyanin-degrading enzymes solution (Vaknin et al., 2005).

To determine the anthocyanin degradation activity in each sample, anthocyanins were extracted from the pericarps of ‘Mavras’ and ‘Tequila’ fruit in 10% (v/v) acetic acid and dried in an evaporator, following which the residues were resuspended in 20 mM sodium acetate buffer (pH 5). A reaction mixture containing 1 mL of anthocyanin solution, 1 mL of anthocyanin-degrading enzymes (1 mg·mL⁻¹), and 1.17 µL of 30% H₂O₂ (Fujifilm Wako Pure Chemical) was then divided into two 1-mL aliquots, and 1 mL of 6 M acetic acid (Fujifilm Wako Pure Chemical) was added to the first sample immediately and to the second sample after 3 min to stop the reaction. The anthocyanin contents in both samples were then measured by HPLC as described previously. Anthocyanin degradation was determined as the decrease in anthocyanin content per minute at room temperature. Three control reaction mixtures were also used, which contained 1) 1 mL of anthocyanin solution and 1 mL of 20 mM sodium acetate (pH 5), 2) 1 mL of anthocyanin solution and 1 mL of anthocyanin-degrading enzymes (1 mg·mL⁻¹), 3) 1 mL of anthocyanin solution and 1.17 µL of 30% H₂O₂, and 4) 1 mL of anthocyanin solution, 1 mL of anthocyanin-degrading enzymes (1 mg·mL⁻¹) denatured by boiling for 10 min, and 1.17 µL of 30% H₂O₂.

STATISTICAL ANALYSIS. Except for zeaxanthin and capsorubin analysis, the significance of differences among the samples was analyzed by the Tukey–Kramer test. In the Tukey–Kramer test, we performed the analysis of variance by using Microsoft Excel software (Microsoft, Redmond, WA) to calculate the *q* value. *q* boundary values were determined at the significance level of *P* < 0.05. The significance test was performed by comparing the *q* value with the *q* boundary value. In zeaxanthin and capsorubin analysis, the significance of differences among the samples was analyzed by Student’s *t* test. We performed Student’s *t* test with a *P* value of <0.05 considered statistically significant.

Results

CHANGES IN ANTHOCYANIN, CINNAMIC ACID DERIVATIVE, AND FLAVONOID CONTENTS. In Expt. 1, no anthocyanins were detected in ‘Mavras’ at 10 DAP, but they became detectable at 15 DAP (Fig. 2A). The anthocyanin content then reached a maximum at 20 DAP (192.0 nmol·g⁻¹), following which it decreased to almost half at 40 DAP and became almost undetectable again at 60 DAP. Similar profiles were observed for ‘Mavras’ in Expt. 2 and ‘Tequila’ in Expts. 1 and 2 (Fig. 2A and B). Conversely, the contents of cinnamic acid derivatives were highest at 50 DAP, whereas those of flavonoids were highest at 15 DAP (Table 1). Both cinnamic acid derivatives and flavonoids were present at much lower concentrations than anthocyanins at 15–50 DAP.

IDENTIFICATION OF ANTHOCYANINS. Nasunin (delphinidin-3-p-coumaroyl-rutinoside-5-glucoside) is a major anthocyanin pigment present in the pericarp of eggplant of a Japanese cultivar and responsible for the purple coloration (Azuma et al., 2008). A fruit of a Japanese eggplant cultivar was commercially purchased, and anthocyanin was extracted from its pericarps in 10% (v/v) acetic acid. Thereafter, the extract was used as a standard of nasunin.

Fig. 2. Content of (A, B) anthocyanins, (C, D) chlorophylls, and (E, F) carotenoids in fruit of the paprika cultivars Mavras and Tequila at the indicated number of days after pollination (DAP) in Expts. 1 (A, C, E) and 2 (B, D, F). Data were recorded on a fresh weight basis and are mean $\pm$ SE (A-B: $n = 3$ at 10, 50, and 60 DAP and $n = 6$ at 15, 20, and 40 DAP; C-F: $n = 3$). Data for the anthocyanin contents were obtained from Table 1, whereas those for the chlorophyll contents are the sum of chlorophylls *a* and *b* from Table 2 and those for the carotenoid contents are the sum of β -carotene, lutein, zeaxanthin, violaxanthin, capsanthin, and capsorubin from Table 3. Asterisks indicate significant differences between 'Mavras' and 'Tequila' fruit at the same DAP (Student's *t* test at $P < 0.05$).

First, we compared the properties of nasunin of a Japanese eggplant and the anthocyanins of 'Tequila'. In the TLC analyses developed with solvents of 10% acetic acid and *n*-butanol/acetic acid/distilled water at a 4:1:2 (v/v/v), the R_f values coincided at 0.47. In the HP1100 HPLC analyses, the retention times coincided at 17.3 min. In absorption spectra recorded in the HPLC, the $\lambda_{\max}$ values practically coincided [280, 300, 310 (shoulder), and 532 nm for nasunin; 280, 300, 310 (shoulder), and 530 nm for the anthocyanins of 'Tequila']. Because of the coincidence of these properties, the main anthocyanins of 'Tequila' were identified to be nasunin, so that it was used as another nasunin standard.

Second, we compared the properties of nasunin and the anthocyanins of 'Mavras'. In the TLC analyses developed with a solvent of 10% acetic acid, the R_f values coincided at 0.47. In the TLC analyses developed with a solvent of *n*-butanol/acetic

acid/distilled water in a ratio of 4:1:2 (v/v/v), the R_f values coincided at 0.47 as well. In EZChrom Elite HPLC analyses, the retention times practically coincided (19.9 min for nasunin and 20.0 min for the anthocyanins of 'Mavras'). Because of the coincidence of these properties, the main anthocyanins of 'Mavras' were also identified to be nasunin. We noted that nasunin was a unique main anthocyanin present in the pericarp of the fruit of both 'Mavras' and 'Tequila' through all growth stages.

CHANGES IN CHLOROPHYLL CONTENTS. In Expt. 1, chlorophylls were detected in 'Mavras' at 10 DAP at a concentration of $24.1 \mu\text{mol}\cdot\text{g}^{-1}$ (Fig. 2C). The total chlorophyll contents then slightly increased to reach a maximum concentration of $36.6 \mu\text{mol}\cdot\text{g}^{-1}$ at 20 DAP, following which they decreased to a low level ($5.0 \mu\text{mol}\cdot\text{g}^{-1}$) at 50 DAP. Similar profiles were observed for 'Tequila' in Expts. 1 and 2 (Fig. 2C and D). However, a different profile was found for 'Mavras' in Expt. 2, with the contents slightly decreasing from 10 to 40 DAP and then sharply decreasing at 50 DAP.

CHANGES IN CAROTENOID CONTENTS. In Expt. 1, the carotenoid contents in 'Mavras' remained at a trace level at 10 to 40 DAP, following which they slightly increased at 50 DAP and then significantly increased to $924.8 \text{ nmol}\cdot\text{g}^{-1}$ at 60 DAP (Fig. 2E). Similar profiles were observed in 'Mavras' in Expt. 2 and in 'Tequila' in Expts. 1 and 2 (Fig. 2E and F).

IDENTIFICATION OF CHLOROPHYLLS AND CAROTENOIDS. Chlorophylls and carotenoids that

were extracted from the fruit of 'Mavras' and 'Tequila' were compared with standard samples. In the HPLC analyses, both the retention times and the $\lambda_{\max}$ values of the compounds that were extracted from these two cultivars matched those of standard samples of chlorophyll *a*, chlorophyll *b*, β -carotene, lutein, zeaxanthin, violaxanthin, capsanthin, and capsorubin, resulting in the identification and quantification of these compounds (Supplemental Table 2).

In Expt. 1, contents of chlorophyll *a* were higher than those of chlorophyll *b* at 10–50 DAP (Table 2) in 'Mavras' and 'Tequila'. Contents of both chlorophyll *a* and chlorophyll *b* in 'Mavras' were higher than those of 'Tequila' at 10–50 DAP. Similar profiles were observed in Expt. 2.

In Expt. 1, the major carotenoids present in 'Mavras' at 60 DAP were capsanthin (53.4%), zeaxanthin (17.3%), β -carotene (14.0%), and capsorubin (13%; Table 3). A similar composition

Table 1. Contents of cinnamic acid derivatives, flavonoids, and anthocyanins (nasunin) in fruits of the paprika cultivars Mavras and Tequila at different numbers of days after pollination (DAP) in Expts. 1 and 2. Data were recorded on a fresh weight basis.

		DAP					
Pigments	Cultivar	10	15	20	40	50	60
Expt. 1 [pmol·g ⁻¹ (mean ± SE)] ^z							
Cinnamic acid derivatives	Mavras	2.0 ± 0.6 b ^y	4.4 ± 0.5 b	1.8 ± 0.4 b	13.1 ± 5.6 a	15.4 ± 0.5 a	12.1 ± 1.8 a
	Tequila	0.9 ± 0.5 b	0.5 ± 0.1 b	1.2 ± 0.3 b	11.3 ± 4.2 a	16.2 ± 2.6 a	10.1 ± 0.9 a
Flavonoids	Mavras	0.2 ± 0.1 b	0.4 ± 0.3 b	1.3 ± 0.4 a	0.2 ± 0.0 b	n.d.	n.d.
	Tequila	0.2 ± 0.0 c	4.9 ± 1.3 a	4.3 ± 0.6 a	0.2 ± 0.1 c	1.6 ± 0.3 b	1.2 ± 0.2 b
Anthocyanins	Mavras	n.d.	127.4 ± 12.8 b	192.0 ± 21.8 a	89.9 ± 34.3 b	8.2 ± 3.2 c	2.6 ± 0.2 c
	Tequila	n.d.	61.2 ± 6.4 b	233.0 ± 33.6 a	61.5 ± 11.5 b	25.0 ± 9.3 c	17.7 ± 2.4 d
Expt. 2 [pmol·g ⁻¹ (mean ± SE)]							
Cinnamic acid derivatives	Mavras	0.8 ± 0.1 b ^z	1.8 ± 0.6 b	1.4 ± 0.1 b	7.0 ± 2.3 a	10.4 ± 5.6 a	7.0 ± 0.8 a
	Tequila	0.2 ± 0.0 c	0.8 ± 0.2 c	1.5 ± 0.3 c	8.8 ± 2.6 b	15.4 ± 3.1 a	12.1 ± 1.8 a
Flavonoids	Mavras	0.1 ± 0.0 c	1.8 ± 0.6 a	1.0 ± 0.5 a	0.9 ± 0.3 b	0.7 ± 0.1 b	0.8 ± 0.0 b
	Tequila	0.6 ± 0.1 c	5.4 ± 1.8 a	2.3 ± 1.0 a	3.7 ± 0.5 a	2.5 ± 1.0 ab	1.6 ± 0.1 b
Anthocyanins	Mavras	n.d.	79.4 ± 12.7 b	165.2 ± 22.8 a	53.3 ± 30.4 b	17.8 ± 12.5 c	n.d.
	Tequila	n.d.	90.6 ± 11.0 b	214.7 ± 19.0 a	46.4 ± 6.2 c	6.1 ± 0.8 d	8.7 ± 7.1 d

^zn = 3 at 10, 50, and 60 DAP; n = 6 at 15, 20, and 40 DAP; n.d. = not detected.

^yDifferent lowercase letters within the same row indicate significant differences (Tukey–Kramer test at *P* < 0.05).

Table 2. Chlorophyll *a* and *b* contents of the fruits of the paprika cultivars Mavras and Tequila at different number of days after pollination (DAP) in Expts. 1 and 2. Data were recorded on a fresh weight basis.

Pigments	Cultivar	DAP					
		10	15	20	40	50	60
Expt. 1 [$\mu\text{mol}\cdot\text{g}^{-1}$ (mean $\pm$ SE)] ^z							
Chlorophyll <i>a</i>	Mavras	16.7 $\pm$ 1.4 b ^y	21.0 $\pm$ 2.2 ab	25.3 $\pm$ 2.9 a	11.1 $\pm$ 2.9 c	4.4 $\pm$ 1.6 d	n.d.
	Tequila	9.5 $\pm$ 1.3 a	10.4 $\pm$ 1.6 a	11.3 $\pm$ 1.8 a	2.7 $\pm$ 0.5 b	0.6 $\pm$ 0.6 c	n.d.
Chlorophyll <i>b</i>	Mavras	7.4 $\pm$ 2.8 a	9.2 $\pm$ 2.3 a	11.0 $\pm$ 1.8 a	2.1 $\pm$ 1.1 b	1.1 $\pm$ 0.2 b	n.d.
	Tequila	4.9 $\pm$ 0.3 b	5.9 $\pm$ 0.7 ab	7.0 $\pm$ 1.0 a	1.8 $\pm$ 0.2 c	0.3 $\pm$ 0.2 d	n.d.
Expt. 2 [$\mu\text{mol}\cdot\text{g}^{-1}$ (mean $\pm$ SE)]							
Chlorophyll <i>a</i>	Mavras	34.6 $\pm$ 2.6 a	29.9 $\pm$ 2.2 ab	25.2 $\pm$ 1.8 b	19.3 $\pm$ 7.3 c	4.1 $\pm$ 0.2 d	n.d.
	Tequila	2.6 $\pm$ 0.6 b	4.3 $\pm$ 1.0 a	6.1 $\pm$ 1.4 a	0.4 $\pm$ 0.1 b	0.2 $\pm$ 0.0 b	n.d.
Chlorophyll <i>b</i>	Mavras	11.3 $\pm$ 3.4 a	11.0 $\pm$ 2.8 a	10.7 $\pm$ 2.2 a	10.1 $\pm$ 3.5 a	2.0 $\pm$ 0.9 b	n.d.
	Tequila	0.8 $\pm$ 0.1 a	1.1 $\pm$ 0.3 a	1.3 $\pm$ 0.5 a	0.1 $\pm$ 0.0 b	0.1 $\pm$ 0.0 b	n.d.

^zn = 3; n.d. = not detected.

^yDifferent lowercase letters within the same row indicate significant differences (Tukey–Kramer test at *P* < 0.05).

of pigments was also found in ‘Tequila’ at 60 DAP in Expt. 1, and the same four compounds were the major carotenoids in both cultivars at 60 DAP in Expt. 2. Thus, the developing fruit of both cultivars showed similar changes in pigment contents in both experiments.

EXPRESSIONS OF ANTHOCYANIN BIOSYNTHESIS-RELATED GENES AND TRANSCRIPTION FACTOR GENES. In both experiments, ‘Mavras’ and ‘Tequila’ showed similar changes in the expression profiles for all of the analyzed genes (Figs. 3 and 4). All genes except *WDR* showed increased expression levels from 10 DAP to reach a maximum at 15 or 20 DAP, following which their expressions decreased to almost undetectable levels at 40 to 60 DAP.

ANTHOCYANIN DEGRADATION. No anthocyanin degradation was detected in the control solution containing only anthocyanin (data not shown). For both cultivars, anthocyanin degradation was significantly higher in the presence of both enzymes extracted from the pericarps of the fruit at 40 DAP and H₂O₂ than in the presence of the enzyme extract alone, H₂O₂ alone, or boiled enzyme extract mixed with H₂O₂ (Fig. 5). Anthocyanin degradation in the presence of both H₂O₂ and enzyme extract from the

fruit remained almost constant at 10 to 20 DAP, increased at 40 DAP, and then leveled off at 50 and 60 DAP for both cultivars in both experiments (Fig. 6).

Discussion

In this study, we detected the anthocyanin nasunin in the fruit of two paprika cultivars Mavras and Tequila, supporting the findings of previous studies on purple paprika cultivars (Aza-González and Ochoa-Alejo, 2012; Lightbourn et al., 2008). In addition, we found that the difference in coloration depth between these two cultivars could be explained by the higher chlorophyll content in the dark purple fruit of ‘Mavras’ than that in the lilac fruit of ‘Tequila’, as shown by Lightbourn et al. (2008). At 10 to 20 DAP, the orange pigment β-carotene and the yellow pigments violaxanthin and lutein were detected in both cultivars, whereas the red pigments capsanthin and capsorubin and the yellow pigment zeaxanthin were not (Table 3). Similarly, Lightbourn et al. (2008) showed that the violet fruit of the true-breeding paprika genotype G05C74-12 did not contain capsanthin or capsorubin. By contrast, at 40, 50, and 60 DAP, the fruit of both cultivars analyzed in this study accumulated capsanthin, with this becoming the main carotenoid by 60 DAP. These findings indicate that complex changes in the contents of nasunin, chlorophylls, and carotenoids determine the color changes in the developing fruit of these paprika cultivars. At 10 DAP, green fruit contained chlorophylls but no anthocyanins and low levels of carotenoids; at 15 and 20 DAP, the fruit were dark purple or lilac because of both nasunin and chlorophylls; and at 40 to 60 DAP, fruit of both cultivars became red because of a decrease in the anthocyanin and chlorophyll contents and the accumulation of carotenoids, particularly capsanthin.

Table 3. Carotenoid contents of the fruits of the paprika cultivars Mavras and Tequila at different number of days after pollination (DAP) in Expts. 1 and 2. Data were recorded on a fresh weight basis.

Pigments	Cultivar	DAP					
		10	15	20	40	50	60
		Expt. 1 [nmol·g ⁻¹ (mean ± SE)] ^z					
β-carotene	Mavras	7.3 ± 1.8 c ^y	30.2 ± 10.3 b	53.1 ± 18.8 b	30.0 ± 15.4 b	59.5 ± 4.6 b	129.0 ± 22.5 a
	Tequila	6.1 ± 2.1 c	6.2 ± 1.3 c	5.0 ± 0.5 c	9.8 ± 5.0 c	53.2 ± 2.6 b	117.8 ± 31.1 a
Lutein	Mavras	2.6 ± 0.4 a	5.4 ± 1.9 a	8.2 ± 3.5 a	1.2 ± 1.1 a	1.2 ± 0.9 a	2.9 ± 0.7 a
	Tequila	1.4 ± 0.2 a	1.6 ± 0.6 a	1.8 ± 1.1 a	0.4 ± 0.2 a	0.9 ± 0.3 a	1.4 ± 0.6 a
Zeaxanthin	Mavras	n.d.	n.d.	n.d.	n.d.	40.3 ± 11.9 b	160.1 ± 16.5 a
	Tequila	n.d.	n.d.	n.d.	n.d.	37.8 ± 5.4 b	170.8 ± 31.1 a
Violaxanthin	Mavras	3.0 ± 1.0 b	3.5 ± 1.5 b	3.9 ± 1.9 b	12.3 ± 3.5 a	17.6 ± 7.0 a	15.5 ± 6.5 a
	Tequila	2.8 ± 0.8 b	1.8 ± 0.5 b	0.6 ± 0.2 b	1.7 ± 1.1 b	2.8 ± 1.0 b	6.9 ± 0.5 a
Capsanthin	Mavras	n.d.	n.d.	n.d.	29.8 ± 6.8 c	122.3 ± 12.1 b	494.2 ± 55.3 a
	Tequila	n.d.	n.d.	n.d.	2.2 ± 0.3 c	129.2 ± 48.6 b	330.5 ± 53.3 a
Capsorubin	Mavras	n.d.	n.d.	n.d.	n.d.	62.8 ± 8.1 b	123.1 ± 20.5 a
	Tequila	n.d.	n.d.	n.d.	n.d.	31.8 ± 5.5 b	53.3 ± 4.7 a
		Expt. 2 [nmol·g ⁻¹ (mean ± SE)]					
β-carotene	Mavras	48.1 ± 22.5 b	48.8 ± 14.3 b	49.4 ± 6.0 b	47.9 ± 14.8 b	96.0 ± 31.3 b	431.7 ± 63.2 a
	Tequila	16.0 ± 4.3 b	11.4 ± 3.3 b	6.9 ± 2.4 b	11.7 ± 2.4 b	49.2 ± 16.2 b	248.8 ± 145.2 a
Lutein	Mavras	4.7 ± 2.8 a	4.8 ± 2.9 a	5.0 ± 1.6 a	1.1 ± 0.4 a	1.4 ± 0.8 a	3.6 ± 1.4 a
	Tequila	1.5 ± 0.4 a	1.2 ± 0.3 a	0.9 ± 0.3 a	0.2 ± 0.1 a	1.3 ± 0.9 a	4.5 ± 2.8 a
Zeaxanthin	Mavras	n.d.	n.d.	n.d.	n.d.	30.9 ± 14.8 b	218.6 ± 21.0 a
	Tequila	n.d.	n.d.	n.d.	n.d.	54.1 ± 11.7 b	192.9 ± 7.9 a
Violaxanthin	Mavras	5.0 ± 1.3 b	9.9 ± 2.2 b	14.8 ± 3.1 b	15.3 ± 6.2 b	29.7 ± 12.0 b	36.2 ± 8.4 a
	Tequila	3.3 ± 1.6 b	3.0 ± 1.4 b	2.7 ± 1.3 b	5.3 ± 1.8 b	24.4 ± 15.6 b	70.8 ± 34.7 a
Capsanthin	Mavras	n.d.	n.d.	n.d.	51.7 ± 4.3 b	41.4 ± 10.8 b	854.4 ± 47.4 a
	Tequila	n.d.	n.d.	n.d.	9.0 ± 3.9 b	19.0 ± 9.2 b	608.7 ± 48.0 a
Capsorubin	Mavras	n.d.	n.d.	n.d.	n.d.	32.9 ± 12.4 b	274.9 ± 36.2 a
	Tequila	n.d.	n.d.	n.d.	n.d.	63.4 ± 14.4 b	216.4 ± 24.8 a

^zn = 3; n.d. = not detected.

^yDifferent lowercase letters within the same row indicate significant differences (Tukey–Kramer test for β-carotene, lutein, violaxanthin, and capsanthin; Student's *t* test for zeaxanthin and capsorubin; *P* < 0.05 for both tests).

In addition, the expressions of anthocyanin biosynthesis-related genes in the fruit of ‘Mavras’ and ‘Tequila’ increased during the unripe stage but then drastically decreased during the ripe stage, which agrees with previous findings for the purple paprika cultivars Arvol and Uvilla (Aza-González et al., 2013). These results suggest that anthocyanin is only synthesized during the unripe stage in purple paprika cultivars. Furthermore, the finding that cinnamic acid derivatives and flavonoids were present at lower concentrations than nasunin (Table 1) indicates that nasunin is biosynthesized without the accumulation of related compounds positioned upstream in the biosynthetic pathway.

It is understood that a complex of the transcription factors *MYB*, *bHLH*, and *WDR* is required for the expression of anthocyanin biosynthesis-related genes. It has previously been demonstrated that lack of any of these transcription factors inhibits anthocyanin biosynthesis in several *Capsicum* spp. (Aguilar-Barragán and Ochoa-Alejo, 2014; Lu et al., 2018; Stommel et al., 2009; Zhang et al., 2015), and our data supported this (Figs. 3 and 4). Anthocyanin biosynthesis-related genes were expressed and anthocyanin was biosynthesized, resulting in an increase in anthocyanin contents in the fruit at 15 and 20 DAP, so that *MYB*, *bHLH*, and *WDR* were possibly expressed at sufficient levels at these stages. Under the constitutive expression of *WDR*, the expressions of the anthocyanin biosynthesis-related genes increased with

increasing expressions of *MYB* and *bHLH* at 15 or 20 DAP and ceased with decreasing expressions of *MYB* and *bHLH* after 40 DAP. We suggest that *MYB* and *bHLH* may positively regulate the transcription of anthocyanin biosynthesis-related genes in the paprika fruit.

Liu et al. (2018) recently proposed that anthocyanins are degraded by peroxidases in solanaceous plants, using anthocyanin degradation in *Brunfelsia calycina* Hook. flowers as an example (Vaknin et al., 2005; Zipor et al., 2015). However, to the best of our knowledge, the enzymatic degradation of anthocyanins in the fruit of solanaceous crops, such as paprika, tomato (*Solanum lycopersicum* L.), and eggplant, has not previously been studied (Sakamura and Obata, 1963), and the suggestion of Liu et al. (2018) has not been tested. In the present study, we found that anthocyanin degradation was highest in the presence of both enzyme extract and H₂O₂ (Fig. 5). Fang et al. (2015) and Zipor et al. (2015) argued that peroxidase requires H₂O₂ and that polyphenol oxidase requires O₂ for the anthocyanin degradation reaction. Furthermore, Barbagallo et al. (2007) showed that β-glucosidase hydrolyzes anthocyanin. However, this enzyme could not be concerned with deletion of coloration in plant tissues because this reaction generates anthocyanidin, i.e., a chromophore of the anthocyanin and could not delete coloration alone. Therefore, it is likely that anthocyanin degradation in purple paprika fruit is mediated by peroxidases.

Fig. 3. Relative expression levels of anthocyanin biosynthesis-related genes and transcription factor genes in fruit of the paprika cultivars Mavras and Tequila at the indicated numbers of days after pollination (DAP) in Expt. 1. Data are means $\pm$ SE ($n = 3$ at 10, 50, and 60 DAP; $n = 6$ at 15, 20, and 40 DAP). Different letters indicate significant differences among DAPs for each cultivar (Tukey-Kramer test, $P < 0.05$). *PAL* = phenylalanine ammonia-lyase, *CHS* = chalcone synthase, *CHI* = chalcone isomerase, *F3H* = flavanone 3-hydroxylase, *F3'5'H* = flavonoid 3'-5'-hydroxylase, *DFR* = dihydroflavonol 4-reductase, *ANS* = anthocyanidin synthase, *UFGT* = UDP glucose: flavonoid 3-O-glucosyltransferase, *bHLH* = basic helix-loop-helix, and *WDR* = WD40-repeat.

The anthocyanin content of plant tissues and organs is controlled by the anthocyanin turnover, as demonstrated for mature flowers of *Petunia $\times$ hybrid* Vilm., mustard seedlings (*Sinapis alba* L.), and grape fruit cell cultures (*Vitis vinifera* L. cv. Gamay Red) (Jonsson et al., 1984; Sinilal et al., 2011; Zenner and Bopp, 1987). Our analysis revealed that at 15 and 20 DAP, the anthocyanin content of the paprika fruit increased and the fruit turned purple, despite the degradation of

anthocyanins by peroxidases, because of increased expressions of the *MYB* and *bHLH* genes, which upregulated the expressions of anthocyanin biosynthesis-related genes. However, at 40 to 60 DAP, the purple color faded because of the high anthocyanin degradation activity and the decreased expressions of the *MYB* and *bHLH* genes resulting in reduced expressions of the anthocyanin biosynthesis-related genes.

Fig. 4. Relative expression levels of anthocyanin biosynthesis-related genes and transcription factor genes in the fruit of the paprika cultivars Mavras and Tequila at the indicated number of days after pollination (DAP) in Expt. 2. See Fig. 3 for additional details.

Furthermore, the contents of green pigments (chlorophylls) decreased and the contents of red pigments (carotenoids, particularly capsanthin) increased at the ripening stage. However, these timings did not completely coincide with the timing of anthocyanin degradation. At 40 DAP, when expression of the anthocyanin biosynthesis-related genes *MYB* and *bHLH* had decreased and anthocyanin degradation activity had increased, the chlorophyll contents had not fully declined and the carotenoid contents had not yet increased. Therefore, it appears that the content of each pigment is individually regulated, suggesting that each single, double, and triple color combina-

tion of purple, green, and/or red could be freely expressed in the mature fruit of paprika.

The first step toward breeding paprika cultivars with purple fruit at the ripe stage is lowering anthocyanin degradation or a continued expression of the anthocyanin regulatory genes (*MYB* and *bHLH*) throughout the ripening process, as this would allow the synthesized anthocyanin content to be retained. One effective method for achieving this may be to screen for cultivars with a lower degradation activity at the ripening stage among the general cultivars that are currently grown. By crossing such fruit cultivars with a lower anthocy-

Fig. 5. Anthocyanin degradation by enzyme extracts from the fruit of the paprika cultivars Mavras and Tequila. Reaction mixture contained anthocyanin and enzyme extract from the pericarps of the paprika fruit at 40 d after pollination (AE); anthocyanin and H₂O₂ (AH); anthocyanin, enzyme extract, and H₂O₂ (AEH); or anthocyanin, boiled enzyme extract, and H₂O₂ (AboEH). Δ anthocyanin content is the difference in anthocyanin content before and after the reaction. Data are means $\pm$ SE (n = 3 for AE, AH, and AboEH; n = 6 for AEH). Different letters indicate significant differences among the reaction mixtures for the same cultivar (Tukey–Kramer test at $P < 0.05$).

Fig. 6. Anthocyanin degradation by enzyme extracts from the fruit of the paprika cultivars Mavras and Tequila in the presence of H₂O₂ at different number of days after pollination (DAP) in Expts. 1 and 2. Data are means $\pm$ SE (n = 3 at 10, 15, and 20 DAP; n = 6 at 40, 50, and 60 DAP). Δ anthocyanin content is the difference in anthocyanin content before and after the reaction. Different letters indicate significant differences among DAP for each cultivar (Tukey–Kramer test at $P < 0.05$).

anin degradation activity at the ripening stage, it is expected to grow cultivars with fruit that develop purple color even at the completely ripened stage.

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Supplemental Table 1. Primers used for quantitative polymerase chain reaction (qPCR).

Gene ^z	Forward sequence (5'-3')	Reverse sequence (5'-3')
PAL	ATTGATTTTGTCAAGAAATCAATTC	GCTCCACTTTAGCCCCAC
CHS	GTTGCTTTGCTGGTGAAC	CACTTGGGCCACGGAAAGTA
CHI	AGTTCCTGGCACTGGGAATG	CTTTGCGGCAGGTGAAACTC
F3H	GCCAAGGAGCAGCAGATACA	TTCCTCAATGGGCATGGATTC
F3'5'H	GATGGGGTGGCCGGTGATTG	GCCACCACAACGCGCTCG
DFR	GCCATTCAAGGCTGTCAAGG	AACATTCCCCTGACCGTTGG
ANS	CAAATGCCCAACAACCAGAACTAGC	CGCACTTTGCAGTTACCCACTTTC
UGFT	ACCCCTATTGGTTGTGAAGCC	ACCTATTTCTCCTCTGCCTC
MYB	AGATTGCCGGGAAGAACAGCAAAC	TTGCACTTGATGAGAAGGTCCGAG
bHLH	CAATGGAGCTATAAAGACTAGGAA	GGAAAAGAGAAAAGAACACACATG
WDR	GTGTGAATGCTATTGCTTGG	GGAGGAGGACCACTGAAG
UBIQUITIN	TGTCCATCTGCTCTCTGTTG	CACCCCAAGCACAATAAGAC

^zPAL = phenylalanine ammonia-lyase; CHS = chalcone synthase; CHI = chalcone isomerase; F3H = flavanone 3-hydroxylase; F3'5'H = flavonoid 3'5'-hydroxylase; DFR = dihydroflavonol 4-reductase; ANS = anthocyanidin synthase; UFGT = UDP glucose: flavonoid 3-O-glucosyltransferase; bHLH = basic helix-loop-helix; WDR = WD40-repeat.

Supplemental Table 2. Retention time and spectral data for chlorophylls and carotenoids that were separated by high-performance liquid chromatography from the reference compounds and extracts from the fruit of the paprika cultivars Mavras and Tequila at 20 d after pollination (DAP) (chlorophylls) or 60 DAP (carotenoids).

Compound		Retention time (min)	$\lambda_{\max}$ (nm)	
Chlorophylls				
Chlorophyll <i>a</i>	Reference	34.2	430	
	Mavras	34.8	432	
	Tequila	34.6	432	
Chlorophyll <i>b</i>	Reference	26.8	452	
	Mavras	27.2	450	
	Tequila	27.1	450	
Carotenoids				
β -carotene	Reference	69.4	451	480
	Mavras	67.6	451	480
	Tequila	67.9	450	479
Lutein	Reference	30.8	445	475
	Mavras	31.1	446	475
	Tequila	31.1	446	475
Zeaxanthin	Reference	31.2	440	470 500
	Mavras	31.8	440	470 502
	Tequila	31.7	441	470 501
Violaxanthin	Reference	15.4	421	472 505
	Mavras	15.5	421	473 503
	Tequila	15.1	421	473 504
Capsanthin	Reference	29.8	470	500
	Mavras	30.1	469	503
	Tequila	30.1	470	503
Capsorubin	Reference	27.2	450	474 507
	Mavras	27.3	448	477 505
	Tequila	26.8	448	477 505