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Effect of anthocyanin composition in grape skin on anthocyanic vacuolar inclusion development and skin coloration

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Summary

Berry skin coloration, the content and composition of anthocyanin and anthocyanic vacuolar inclusion (AVI) development were investigated in two black cultivars, Cabernet Sauvignon (*Vitis vinifera*) and Pione (*V. vinifera* × *V. labrusca*, 4x), and one red cv., Red Port (*V. vinifera* × *V. labrusca*). Pione had lower L* (lightness of the skin) and higher color index values than Cabernet Sauvignon, indicating that Pione had darker skin color than Cabernet Sauvignon. The two black cultivars had high contents of malvidin, while the major anthocyanidin of Red Port was cyanidin and delphinidin. This difference of anthocyanidin composition was responsible for the red color of Red Port. Whereas the anthocyanidin composition of the two black cultivars showed little difference, the percentage of acylated anthocyanins was markedly higher in Pione than in Cabernet Sauvignon. The diameter of AVIs was similar between Pione and Cabernet Sauvignon, but the density of AVIs was higher in cv. Pione. This difference in AVI development affects grape skin coloration. A comparison of the anthocyanin composition in isolated AVIs and that in whole cell tissue showed that in all three cultivars the percentage of acylated anthocyanins was high in the AVIs. In Pione skins the high percentage of acylated anthocyanins might result in many AVIs to be formed, and might be responsible for the dark coloration.

K e y w o r d s : anthocyanic vacuolar inclusion, anthocyanin, grape coloration, acylation.

A b b r e v i a t i o n s : AVI, anthocyanic vacuolar inclusion; CIRG, color index for red grapes; Mv 3G, malvidin-3-glucoside; Mv 3*p*G, malvidin-3-*p*-coumaryl glucoside; Mv 3AcG, malvidin-3-acetyl-glucoside; Mv 3*p*G5G, malvidin-3-*p*-coumaryl glucoside-5-glucoside; Pn 3G, peonidin-3-glucoside; Dp 3*p*G, delphinidin-3-*p*-coumaryl glucoside,

Introduction

Dark skin coloration is one of the most important quality factors of table and wine grapes. Grape skin color is usually determined by the content and composition of anthocyanins in epidermal and hypodermal cells (Kliewer and Torres 1972, Shiraishi and Watanabe 1994). It is generally accepted that the formation of anthocyanic vacuolar inclusions (AVIs), which are spherules containing anthocyanins at high concentrations, is critical for the dark coloration of grape skin. Nakamura (1993) reported that the formation of AVIs is essential for the dark coloration of Kyoho berry skin. Okamoto *et al.* (2003) reported that the application of large amounts of fertilizer inhibited AVI formation, thereby resulting in poor coloration. The presence of AVIs was confirmed in plant tissues containing anthocyanins, such as radish seedlings (Yasuda and Shinoda 1985), sweet potats (Nozue *et al.* 1997), and rose petals (Gonnet 2003).

As far as we know, there are few reports on the physiological and cytological conditions for AVI formation in the grape skin. In this study, the chemical properties of anthocyanins in the AVIs of black and red grape cultivars were investigated.

Material and Methods

P l a n t m a t e r i a l : Two black cultivars, Cabernet Sauvignon (*Vitis vinifera*) and Pione (*V. vinifera* × *V. labrusca*, 4x), and one red cultivar, Red Port (*V. vinifera* × *V. labrusca*), were used for this study in 2004. Four - six vines of each cultivar were planted in root zone restricted soil beds of which the sides and bottom were wrapped with water-permeable but root-proof polyethylene sheets to prevent root extension from the root zone. Together with irrigation water complete liquid fertilizers, Otsuka Ekihi No. 1 and No. 2, were supplied by a drip irrigation system twice a week. The composition of the liquid fertilizers is reported in a previous paper (Okamoto *et al.* 2003). Twenty berries of average size and similar stage of development were harvested from each grapevine on August 5 for Red Port, August 17 for Pione, and August 31 for Cabernet Sauvignon.

J u i c e a n a l y s e s : The skin of berries was peeled off by hand immediately after sampling. The flesh without seeds was homogenized, centrifuged at 4,000 x g for 5 min, and filtered. Total soluble solids (TSS) and pH were determined by a hand refractometer (Atago N-1α) and a pH meter (Horiba F-22), respectively. Titratable acidity (TA) of the juice was determined by titration with 0.1 N NaOH and expressed as tartaric acid equivalent.

S k i n c o l o r d e t e r m i n a t i o n : Six berries showing similar coloration were chosen from 6 clusters of each cultivar. Berries were wiped clean and skin color was measured with a colorimeter (Nippon Denshoku, NR-3000) as CIE (Commission Internationale de I'Eclairage, International Commission of Illumination) 1976 L*, a*, and b*.

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The L* value represents black to white as the values increase from negative to positive. The a* value represents green to red color as the values increase from negative to positive. The b* value represents blue to yellow color as the values increase from negative to positive.

According to Carreño and Martinez (1995), the color index for red grapes (CIRG) is expressed as CIRG = (180-h) / (L*+C*), where h is the hue angle and C* is chroma.

The CIRG value indicates the degree of skin coloration: high values mean dark coloration.

AVI observation and isolation: Fresh skin discs, 5 mm in diameter and 0.1-0.2 mm thick, were obtained by cutting with a razor blade. They were mounted on a glass slide with distilled water, and covered by a cover glass. Photographs taken under a light microscope were used to count the number and size of AVIs in both epidermal and hypodermal cells.

AVI isolation was performed using the modified method of Conn *et al.* (2003). Ten fresh skin discs, 5 mm in diameter, excised with a razor blade, were soaked in citric phosphate buffer (pH 5.0) containing 1.5 % cellulase (Yakult Pharmaceuticals), 0.6 % maceroenzyme (Yakult Pharmaceuticals), 0.4 % PEG-6000, 5 mM KCl, 2 mM $CaCl_2$, and 0.7 M mannitol. The mixtures were incubated at 25 ℃ for 24 h. After supersonication for 10 s, they were filtered through Miracloth (CALBIOCHEM). The AVIs were finally obtained by centrifugation at 3,000 x g for 10 min.

Anthocyanin analysis: Skin anthocyanins were extracted from 50 mg of frozen skin samples with 50 ml of 50 % acetic acid 3 times for 5 min each. AVI anthocyanins were extracted from isolated AVIs with 50 % acetic acid at room temperature for 6 h. The optical density at 520 nm of skin anthocyanins was determined with a spectrophotometer (Beckmann DU530). To analyze skin anthocyanin and AVI anthocyanin compositions, each extract was filtered through a membrane filter (0.45 μm) and the filtrate was subjected to HPLC (JASCO) at the following conditions: column, STR ODS (250 × 4.6 mm i.d.); column temperature, 35 ℃; flow rate, 0.8 ml min^{-1}; mobile phase, solution A: 1.5 % phosphoric acid, solution B: 1.5 % phosphoric acid + 20 % acetic acid + 25 % AcCN, gradient (solution A %), 0 min 75 %, 0-40 min 15 %; detection at 520 nm. Identification of the detected peaks was based on results of Mochioka *et al.* (1995) and Fujishima and Shiraishi (1997).

Results

Juice composition and skin color: TSS, TA and pH values of juice from each cultivar are shown in Tab. 1. Red Port juice had the highest TSS, whereas Pione juice had the lowest TA and the highest pH.

Berry skin color of each cultivar is expressed as L*, a* and b* values in Tab. 2. The L* value was highest in

Table 1

Harvest date and juice characteristics of three cultivars

Cultivar	Harvest date	TSS[z] (°Brix)	TA ($g \cdot l^{-1}$)	pH
Pione (*V. vinifera* × *V. labrusca*, 4x)	Aug. 17	15.5 b[y]	2.61 b	3.6 a
Cabernet Sauvignon (*V. vinifera*)	Aug. 31	15.2 b	3.41 a	3.1 b
Red Port (*V. vinifera* × *V. labrusca*)	Aug. 5	17.9 a	3.55 a	2.9 b

[z] Represented as tartaric acid equivalent.
[y] Significant difference as determined by Duncan's multiple range test ($p<0.05$) for values within each column.

Table 2

Colorimeteric values of berry skin color[z] in three grape cultivars

Cultivar	Type of skin color	L*	a*	b*	CIRG
Pione	Black	28.8 c[y]	2.9 b	-1.4 b	6.44 a
Cabernet Sauvignon	Black	35.3 a	3.4 b	-7.7 c	3.64 c
Red Port	Red	32.1 b	15.7 a	1.4 a	5.65 b

[z] L* values represent black to white as the value increases from negative to positive; a*: green to red color as the value increases from negative to positive; b*: blue to yellow color as the value increases from negative to positive. CIRG: Color index for red grapes = (180-h)/(L* + C*).
[y] For statistical details see Tab. 1.

Table 3

Total skin anthocyanin content and composition in three grape cultivars

Cultivar	Total anthocyanins (OD_{520})[z]	Main constituent of anthocyanin[y]	Acrylated anthocyanins (%)
Pione	0.62 b[x]	Mv 3*p*G5G, Mv 3*p*G	75.2 a
Cabernet Sauvignon	1.54 a	Mv 3G, Mv 3*p*G	46.3 b
Red Port	0.62 b	Unknown, Dp 3*p*G	46.1 b

[z] Extracted from 50 mg D.W. of skin tissue with 50 ml of 50 % CH_3COOH.
[y] Mv 3*p*G5G: malvidin-3-*p*-coumaryl glucoside-5-glucoside; Mv 3*p*G: malvidin-3-*p*-coumaryl glucoside; Mv 3G: malvidin-3-glucoside; Dp 3*p*G: delphinidin-3-*p*-coumaryl glucoside.
[x] For statistical details see Tab. 1.

Cabernet Sauvignon skins and lowest in Pione skins. The a* value was highest in Red Port skins. The b* value was low in Cabernet Sauvignon skins. These results indicate that the skin color of Pione, Cabernet Sauvignon and Red Port berries is dark black, bright blue and bright red, respectively.

CIRG indicates the degree of skin coloration: high values mean dark coloration. Red Port, a red cultivar, had the lowest value, while the values for Pione were higher than those for Cabernet Sauvignon.

Anthocyanin content and composition: Total anthocyanin content in skins, expressed as OD_{520}, was significantly higher for Cabernet Sauvignon than for Pione or Red Port (Tab. 3). Interestingly, there was no difference in the total anthocyanin content between Pione (black berries) and Red Port (red berries). HPLC analyses of skin extracts revealed that the major anthocyanins in Pione were malvidin-3-*p*-coumarylglucoside-5-glucoside (Mv 3*p*G5G) and malvidin-3-*p*-coumarylglucoside (Mv 3*p*G). In Cabernet Sauvignon, malvidin-3-glucoside (Mv 3G) was the major component, followed by Mv 3*p*G. Four major peaks were found in Red Port: peaks 1-3 were unidentifiable, whereas peak 4 was speculated to be delphinidin-3-*p*-coumarylglucoside (Dp 3*p*G). The percentage of acylated anthocyanins in each cultivar was calculated based on the peak area. In Cabernet Sauvignon and Red Port skins approximately 50 % of total anthocyanins were acylated, while in Pione, 75 % of the total anthocyanins were acylated.

AVI development: AVIs were found in the berry skin of all cultivars; however, the density, size and shape varied depending on the cultivar (Fig. 1). In Pione skin, large AVIs with diameters exceeding 15 µm were observed in both epidermis and hypodermis (Tab. 4). Small AVIs (diameter <10 µm) were also found in abundance. Only a few anthocyanins were located outside the AVIs. In Cabernet Sauvignon skin, large and small AVIs were observed in both epidermis and hypodermis. However, the number of AVIs was smaller than in Pione skins, and anthocyanins seemed to be distributed both inside and outside the AVIs. In the case of Red Port, AVIs existed only in epidermal cells and most of them were smaller than 15 µm. The anthocyanins of Red Port were found in both AVIs and vacuole sap, similar to the Pione skin.

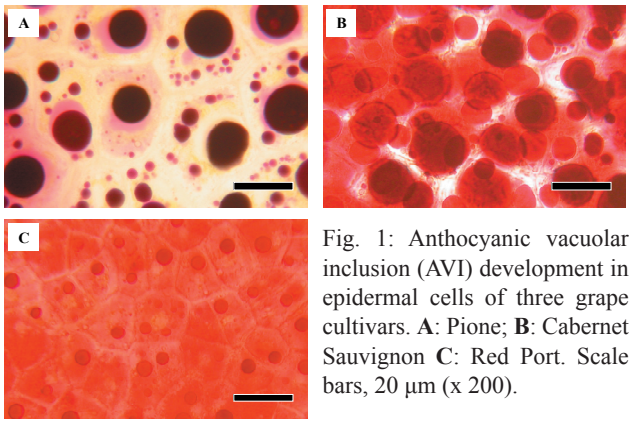

Fig. 1: Anthocyanic vacuolar inclusion (AVI) development in epidermal cells of three grape cultivars. **A**: Pione; **B**: Cabernet Sauvignon **C**: Red Port. Scale bars, 20 µm (x 200).

Anthocyanin composition of AVIs: The composition of anthocyanins extracted from whole skin tissues and those extracted from isolated AVIs was investigated. Fig. 2 shows the HPLC profiles of whole skin tissue (upper) and AVI (lower) anthocyanin extracts from Pione. Peonidin-3-glucoside (Pn 3G) and Mv 3G were detected

Table 4

Number of anthocyanic vacuolar inclusions (AVIs) per mm^2 of epidermal and hypodermal tissue from berry skin

Cultivar	Tissue	AVI diameter (µm)				
		< 5	5-10	10-15	15-20	> 20
Pione	Epidermis	444	381	181	219	141
	Hypodermis	56	37	28	69	84
Cabernet Sauvignon	Epidermis	131	96	158	142	103
	Hypodermis	0	0	0	12	77
Red Port	Epidermis	87	827	83	0	0
	Hypodermis	0	0	0	0	0

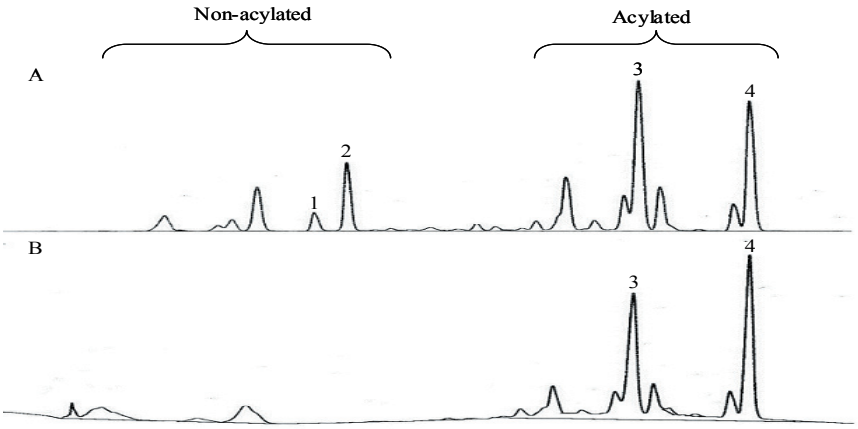

Fig. 2: HPLC profiles of skin anthocyanins in Pione grape berries. A: Anthocyanins extracted from whole skin tissue; B: Anthocyanins extracted from isolated AVIs. Peak 1: petunidin-3-glucoside (Pn 3G); 2: malvidin-3-glucoside (Mv 3G); 3: malvidin-3-p-coumaryl glucoside (Mv 3pG); 4, malvidin-3-p-coumaryl glucoside-5-glucoside (Mv 3pG5G).

in the whole skin tissue extract but not in the AVI extract. In the case of Cabernet Sauvignon (Fig. 3), the most predominant anthocyanin in the whole skin tissue extract was Mv 3G, followed by Mv 3AcG and Mv 3*p*G. In contrast, Mv 3*p*G was the most predominant anthocyanin in the AVI extract. The HPLC profiles of Red Port (Fig. 4) indicated that the whole skin tissue extract had 4 large peaks, two of which were supposed to be non-acylated anthocyanins and the other two, acylated ones. In contrast, the AVI extract contained only two large peaks that were supposed to be acylated anthocyanins. The results indicate that in the three cultivars the AVI extracts contained significantly higher percentages of acylated anthocyanins than the whole skin tissue extracts.

Discussion

HARBORNE (1976) reported that the hydration of the B ring in the anthocyanin molecule is responsible for the bluish color. ARISUMI (1985) concluded that the blue pigmentation of rhododendron petals is influenced by the hydroxylation and methylation of anthocyanin. In grape berries, red cultivars contain high levels of cyanidin and peonidin in skin tissues, whereas black cultivars contain high levels of delphinidin and malvidin (SHIRAISHI and WATANABE 1994). In this experiment, the two black cultivars, Pione and Cabernet Sauvignon, had high levels of malvidin, in agreement with the study of SHIRAISHI and WATANABE (1994). We could not identify the anthocyanins in Red Port skin; however, SHIRAISHI and WATANABE (1994) reported that the main anthocyanidins in the skin pigment of this cultivar are cyanidin and delphinidin. The extent of grape skin coloration is usually dependent on the anthocyanin content (KLIEWER and TORRES 1972). Our present study showed, however, that the anthocyanin contents of Pione and Red Port are not significantly different. This indicates that the difference in skin color between red and black cultivars can be ascribed to the anthocyanidin composition, as shown in this study.

The Pione clusters used in this study had dark skin coloration with visual scores exceeding 8 according to the "Fruit color chart for red, purple and black grapes" (Ministry of Agriculture, Forestry and Fisheries of Japan). Pione berries had lower L* and higher CIRG values than Cabernet Sauvignon indicating that the skin coloration of Pione berries is darker than that of Cabernet Sauvignon (CARREÑO and MARTINEZ 1995). Despite the darker coloration

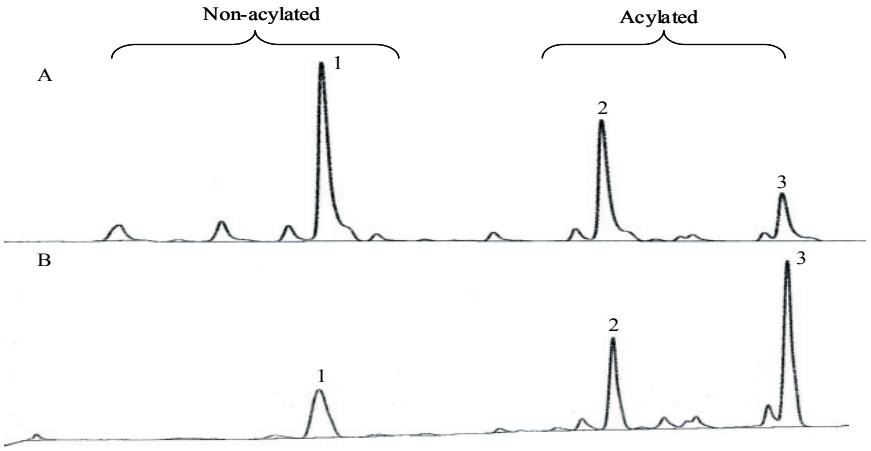

Fig. 3: HPLC profiles of skin anthocyanins in Cabernet Sauvignon grape berries. **A**: Anthocyanins extracted from whole skin tissue; **B**: Anthocyanins extracted from AVIs. Peak 1: malvidin-3-glucoside (Mv 3G); 2: malvidin-3-acetyl glucoside (Mv 3AcG); 3: malvidin-3-p-coumaryl glucoside (Mv3pG).

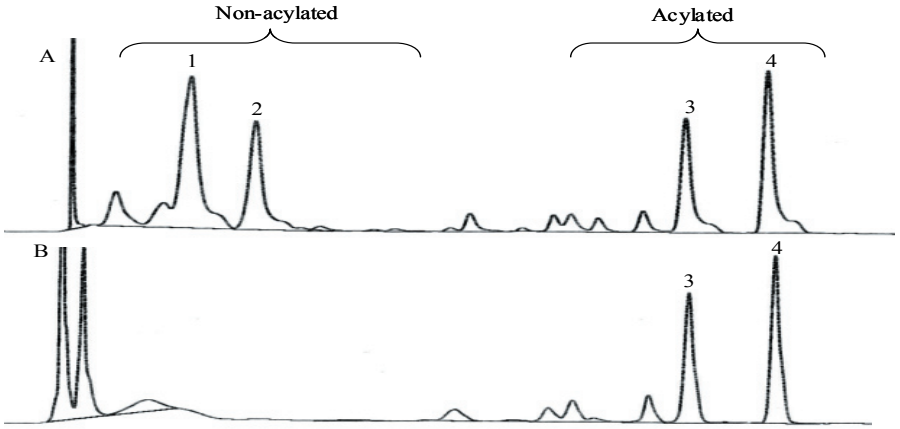

Fig. 4: HPLC profiles of skin anthocyanins in Red Port grape berries. **A**: Anthocyanin extracted from whole skin tissue; **B**: Anthocyanins extracted from AVIs. Peak 1, 2, 3, Unknown; 4, delphinidin-3-p-coumaryl glucoside (Dp 3pG).

of Pione berry skin, however, the anthocyanin content was significantly higher in Cabernet Sauvignon. Because of the similarity in anthocyanidin composition between Pione and Cabernet Sauvignon berry skins, we surmise that factors other than anthocyanidin composition and total anthocyanin content must affect the difference in skin coloration between the two cultivars.

Nakamura (1993) and Okamoto *et al.* (2003) reported that the number and size of AVIs influenced grape berry skin coloration. We noted that AVIs having diameters >15 µm were formed in the epidermal and hypodermal cells of Cabernet Sauvignon berry skin. The size of AVIs in Pione berry skin was similar to that in Cabernet Sauvignon. However, AVI density in both, hypodermis and epidermis, was significantly higher in Pione than in Cabernet Sauvignon. From these results, the difference in skin coloration between the two black cultivars is assumed to be caused by the AVI density.

In grape skin cells where many AVIs had been formed, detectable amounts of anthocyanin are usually found in vacuoles outside the AVIs. We suggest that anthocyanin composition may be a major factor influencing the percentage of anthocyanins in AVIs. HPLC analysis of anthocyanin extracts obtained from isolated AVIs and those from whole skin tissue revealed that significantly higher percentages of acylated anthocyanins that bind with *p*-coumaric acid or acetic acid were available in AVI extracts than in whole skin tissue extracts in all three cvs tested. Conn *et al.* (2003) reported that AVIs contained high percentages of *p*-coumaroylglucosides of cyanidin, peonidin and malvidin in suspension culture cells of *V. vinifera* grapes, which agrees with our result. The high percentage of acylated anthocyanins in Pione skin cells might enable much anthocyanin to be trapped in AVIs, resulting in the further formation of the AVIs.

It is generally accepted that to induce the dark skin coloration of grape berries, the hydroxylation and methylation of anthocyanins, as well as the amount of total anthocyanins, are effective (Kliewer and Torres 1972, Shiraishi and Watanabe 1994). Our study suggests that the high percentage of acylated anthocyanins enhances AVI development, which in turn results in the dark skin coloration of Pione berries. We conclude that the acylation of anthocyanins in grape skin cells is also one of the important factors for the dark skin coloration of grape berries.

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