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Structural Identification of Anthocyanins and Analysis of Concentrations during Growth and Flowering in Buckwheat (*Fagopyrum esculentum* Moench) Petals

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The anthocyanin profiles and variety/breeding-line differences of anthocyanin concentrations in petals of common buckwheat flowers have been studied. Four anthocyanins, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-rhamnoside, and cyanidin 3-*O*-galactosyl-rhamnoside were isolated from the petals of common buckwheat (*Fagopyrum esculentum* Moench), separated using high performance liquid chromatography and identified using reversed-phase liquid chromatography–electrospray ionization–tandem mass spectrometry techniques. In every variety/breeding line tested, cyanidin 3-*O*-rutinoside was detected as the major anthocyanin and the next is cyanidin 3-*O*-glucoside whereas cyanidin 3-*O*-rhamnoside and cyanidin 3-*O*-galactosyl-rhamnoside were trace or not detectable in white and pink flowered buckwheat. Of all the varieties/breeding lines tested, Gan-Chao, a Chinese variety, contained the highest amount of anthocyanins. The largest part of cyanidin moiety was presented as a proanthocyanidin form (PAs–Cy). Anthocyanins and PAs–Cy in petals were increased along with increase of flower development stages. Therefore, fully developed petals of red flowered buckwheat, especially Gan-Chao, are promising as a new anthocyanin-rich material for food processing.

KEYWORDS: Anthocyanins; ESI-MS; flavonoids; flower; buckwheat; petal

INTRODUCTION

Anthocyanins are intensely colored water-soluble pigments, responsible for red, purple, or blue color, that attract consumers to fruits, leaves, and flowers. These compounds can be present in many plant organs such as leaves, stems, and flowers (*1*). Structures of flower anthocyanins were identified in many plants such as carnation (*2*), petunia (*3*), and Liliaceae (*4*). Common buckwheat *(Fagopyrum esculentum* Moench) also contains anthocyanins. Buckwheat anthocyanins were identified only in sprouts; Watanabe (*5*) showed that buckwheat sprouts contained cyanidin 3-*O*-rutinoside as a major anthocyanin. In addition, Kim et al. (*6*) demonstrated that buckwheat sprouts contained at least four anthocyanins, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-galactoside, and cyanidin 3-*O*-galactosyl-rhamnoside. On the other hand, buckwheat contains anthocyanins in its flower. The colors of common buckwheat flowers are generally white, pink, or red. In food processing, petals of red flowered buckwheat are used as materials in tea, juice, and alcohols (*7*) after detoxification of fagopyrin. However until now, no research work has focused on anthocyanin in buckwheat flower. On the other hand, some anthocyanins were present in the hydrolyzed product of proanthocyanidins (PAs) fractions of buckwheat groats and hull (*8*). However, they did not identify it. To investigate anthocyanin in buckwheat flower, identification of anthocyanin in PAs is also important. In addition, no work has focused on the varietal differences of anthocyanin composition and the accumulation pattern of them in different growth stages of flower development. This information is important to select the anthocyanin-rich variety or to determine the harvest time of buckwheat flowers.

In this paper, we identified anthocyanins and anthocyanin in PAs from buckwheat flowers using the high performance liquid chromatography–electrospray ionization–tandem mass spec-

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trometry (HPLC-ESI-MS/MS) technique, and we investigated variety/breeding-line differences and the accumulation pattern of anthocyanins in different growth stages of buckwheat flower.

MATERIALS AND METHODS

Chemicals. Anthocyanins (cyanidin, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, and cyanidin 3-*O*-rhamnoside) for external standards were purchased from Extrasynthèse (Genay, France). HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Plant Materials. Common buckwheat varieties/breeding lines were grown at the experimental field of the National Agricultural and Food Research Organization for the Hokkaido Region in Memuro, Hokkaido, Japan (latitude, 42°53′; longitude, 143°03′). There are only a few summer type varieties/breeding lines of buckwheat (such as Tanno-Hiushinai-r, Sumchanka, and Gan-Chao) that have a red flower. Therefore, in this experiment, we used only five varieties/breeding lines. Buckwheat seeds were sown on June 6, 2006. The petals of four flowers were harvested and stored at −130 °C until used for experiments. To investigate the accumulation pattern of anthocyanin, petals were harvested for each growth stage and were classified according to fresh weights (fw's) as follows: "Stage 1", 0.0–0.4 mg; "Stage 2", 0.4–0.8 mg; "Stage 3", 0.8–1.6 mg; "Stage 4", 1.6–2.6 mg; "Stage 5" (fully developed petal), 2.6–3.6 mg. After "Stage 5", the petals deteriorated easily. Therefore, we did not harvest petals after "Stage 5".

HPLC/ESI-MS Analysis for Anthocyanins. HPLC/ESI-MS analysis was carried out according to the method of Yamazaki et al. (*9*). The anthocyanins were extracted overnight at 22 °C with 1 mL of extraction solvent (MeOH/AcOH/H_2O, 80:0.2:19.8) per 80–100 mg of samples. The extracts were passed through a 0.45 μm filter and applied to an HPLC/ESI-MS system consisting of an Agilent 1100 series LC (Agilent Technologies) coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The positive-ion ESI-MS was performed at a capillary temperature of 365 °C and a voltage of 3.5 kV with N_2 as the sheath gas at a nebulizing pressure of 50 psi. The ion trap MS analysis was carried out with He as the collision gas. The normalized collision energy was set to 30%. HPLC was carried out on a 150 × 2 mm i.d. Cadenza CD-C_{18} column (Imtakt Corporation, Kyoto, Japan) at a flow rate of 0.3 mL/min. To determine the anthocyanin profiles, these compounds were separated using a 0–50 min linear gradient of 0 to 100% solvent A (CH_3CN/H_2O/trifluoroacetyl (TFA), 7.5:92.5:0.1) to solvent B (CH_3CN/H_2O/TFA, 55:45:0.1). The anthocyanin concentrations were determined by the peak areas of the extracted ion chromatogram (EIC, cyanidin 3-*O*-glucoside *m*/*z* 449, $[M + H]^+$; cyanidin 3-*O*-rutinoside, *m*/*z* 595, $[M + H]^+$; cyanidin 3-*O*-rhamnoside *m*/*z* 433, $[M + H]^+$; cyanidin 3-*O*-galactosyl-rhamnoside, *m*/*z* 595, $[M + H]^+$) using a standard curve derived from commercial anthocyanins. As cyanidin 3-*O*-galactosyl-rhamnoside was not commercially available, we used cyanidin 3-*O*-rutinoside instead.

Hydrolysis. The anthocyanins were purified thorough HPLC by preparing fractions corresponding to the anthocyanin peaks using the method described above in the HPLC/ESI-MS Analysis for Anthocyanins section. The isolated anthocyanins were partially hydrolyzed (35 min at 60 °C) in a solution of (MeOH/0.6 N HCl, 50:50) and then characterized using HPLC and comparison to standards.

To determine the PAs–Cy concentration in the nonextractable fraction, first we suspended samples overnight at 22 °C with 1 mL of extraction solvent (MeOH/AcOH/H_2O, 80:0.2:19.8) per 80–100 mg samples to remove the extractable anthocyanins. The centrifuged precipitants were rinsed with the solvent (MeOH/AcOH/H_2O, 80:0.2:19.8) and then suspended to 1 mL of hydrolysis solution (MeOH/6N HCl, 90:10), hydrolyzed at 100 °C for 30 min. The centrifuged supernatants were applied for HPLC using the same method described above. The anthocyanins in the hydrolyzed product were determined by the peak areas of the EIC using a standard curve derived from a standard cyanidin.

Statistical Analysis. The correlation matrix (Pearson correlation coefficient) was calculated using the Microsoft Excel software.

RESULTS AND DISCUSSION

Separation and Structural Identification of Anthocyanins. The HPLC anthocyanin profiles at 512 nm of the petals of red flowered buckwheat (Gan-Chao) show three apparent peaks corresponding to anthocyanin (**Figure 1A**). By comparison of their retention times, MS, and MS/MS spectra to those of the standards, the three anthocyanins were identified as cyanidin 3-*O*-glucoside (**Figure 1 B**), cyanidin 3-*O*-rutinoside (**Figure 1C**), and cyanidin 3-*O*-rhamonoside (**Figure 1D**). On the other hand, the EIC at *m*/*z* 595 gave two major peaks (**Figure 1C**). One peak corresponds to the cyanidin 3-*O*-rutinoside, and the source of the other peak, which had almost the same retention time as the peak A1 (**Figure 1A**), is unknown. To identify the unknown peak, we purified peak A1, partially hydrolyzed, and then analyzed using HPLC by comparison with commercially obtained standards. From the retention times, MS, and MS/MS spectra of the partially hydrolyzed products (data not shown), we conclude that the peak C1 (**Figure 1C**) is cyanidin 3-*O*-galactosyl-rhamnoside. The cyanidin 3-*O*-rutinoside was identified for the first time in buckwheat species whereas the other three anthocyanins, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, and cyanidin 3-*O*-galactosyl-rhamnoside, were also identified in buckwheat sprouts (*6*). In the hydrolyzed product of the nonextractive fraction, only one peak corresponding to cyanidin was detected (data not shown). It indicates that the buckwheat flower contains cyanidin moieties both as "anthocyanin form" and "PAs form (PAs–Cy)".

Varietal Differences of Anthocyanin and PAs–Cyanidin Contents in Buckwheat Flower. To identify anthocyanins in pink or white flowers, we investigated varietal differences of anthocyanin composition and its contents of five buckwheat varieties. **Table 1** shows anthocyanin and PAs–Cy contents of cultivars/breeding lines that have flowers of different colors. The major anthocyanin was the cyanidin 3-*O*-rutinoside, and the next one is the cyanidin 3-*O*-glucoside (except for Nepal native). The cyanidin 3-*O*-galactosyl-rhamnoside and the cyanidin 3-*O*-rutinoside were minor anthocyanins and not detectable in petals of white flowered buckwheat. In every variety/breeding line tested, Gan-Chao, a Chinese variety, contained the highest amount of anthocyanins. Therefore, one can conclude that Gan-Chao is promising as an anthocyanin-rich variety. The anthocyanins found in buckwheat petals are also distributed in other plants. Among them, cyanidin 3-*O*-rutinoside is widely observed in plants such as berries (*10*) or blood oranges (*11*). Cyanidin 3-*O*-glucoside is the major anthocyanin in black soybean seed coats (*12*). It has many antioxidative and anti-inflammatory activities in vitro (*13*), and it prevents obesity and ameliorates hyperglycemia in mice (*14*). Cyanidin 3-*O*-rutinoside, found in blueberry fruit, exhibits inhibitory effects on the migration of human lung cancer cell lines (*15*). Therefore, anthocyanins identified in buckwheat petals may be expected to have similar functions.

Rutinose, the glycosidic entity of cyanidin 3-*O*-rutinoside, is the same glycosidic unit found in rutin, the major flavonol in buckwheat plants. This suggests that cyanidin glycosyltransferases and glycosidases in the cotyledons of tartary buckwheat probably have characteristics, in terms of sugar moiety substrate specificity, similar to those reported for flavonol 3-*O*-glycosydase and flavonoid 3-*O*-glycosyltransferase (*16*–*18*).

In every variety/breeding line, amounts of PAs–Cy were detected (**Table 1**). The amount of PAs–Cy is higher in white or pink flowers than in red flowers (**Table 1**). It is noteworthy that most of the cyanidin moiety was present as a PAs form (**Table 1**). This is the first report to demonstrate the case that

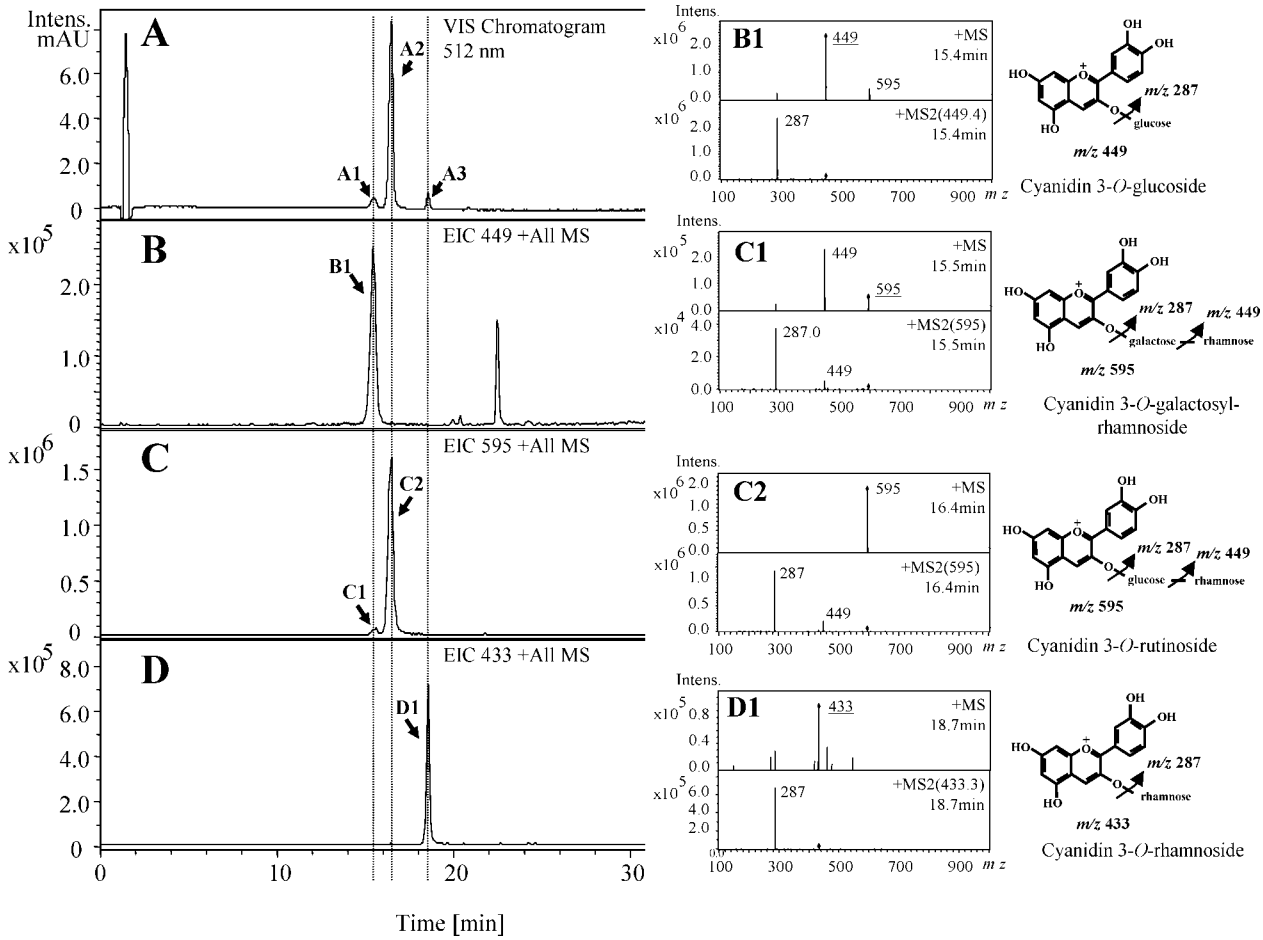

Figure 1. Identification of anthocyanins in petals of red flowered buckwheat. **A**: Anthocyanin profile of petals of red flowered buckwheat (detected at A512). **B**: EIC at *m*/*z* 449. **C**: EIC at *m*/*z* 595. **D**: EIC at *m*/*z* 433.

Table 1. Varietal Differences of Anthocyanin Content in Buckwheat Flower[a]

name	flower color	mg fw/flower	cyanidin 3-*O*-galactosyl-rhamnoside (μg/petals in a flower)	cyanidin 3-*O*-glucoside (μg/petals in a flower)	cyanidin 3-*O*-rutinoside (μg/petals in a flower)	cyanidin 3-*O*-rhamnoside (μg/petals in a flower)	PAs–Cy (μg/petals in a flower)
Kitawasesoba	white	>3.46 ± 0.64	ND	trace	0.06 ± 0.03	ND	32.9 ± 2.7
Tanno-Hiushinai-r	pale pink	4.01 ± 0.27	ND	0.26 ± 0.01	1.16 ± 0.10	0.09 ± 0.02	29.0 ± 1.1
Sumchanka	pale pink	4.73 ± 0.30	trace	0.65 ± 0.37	1.07 ± 0.21	0.19 ± 0.06	38.4 ± 3.6
Nepal native	light pink	3.67 ± 0.23	0.03 ± 0.01	0.02 ± 0.01	1.82 ± 0.87	0.23 ± 0.05	13.8 ± 3.0
Gan-Chao	red	3.26 ± 0.13	0.29 ± 0.21	1.12 ± 0.12	2.95 ± 0.17	0.33 ± 0.11	18.4 ± 1.6

[a] ND: not detectable (mean ± SD, $n = 3$).

the cyanidin moiety was mainly distributed as a PAs form rather than as anthocyanins. In many plants, anthocyanins and PAs share the same synthetic pathway (*19*, *20*). Therefore, the regulation that determines the cyanidin distribution to anthocyanins or PAs–Cy may be an important factor in determining flower colors.

Analysis of Anthocyanins and PAs–Cy Contents during Growth of Red and White Flowered Buckwheat. To investigate the accumulation pattern of anthocyanins and PAs–Cy during flower development, we measured the anthocyanins and PAs–Cy contents at different growth stages of the flower petals of white and red flowered buckwheat (**Table 2**, **Figure 2**). In white flowered buckwheat (**Table 2**, Kitawasesoba), amounts of cyanidin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside were detected after "Stage 2", and they increased along with the growth stages. We could not detect cyanidin 3-*O*-galactosyl-rhamnoside and cyanidin 3-*O*-rutinoside at any growth stage. On the other hand, PAs–Cy was detected at every growth stages, and its contents increased along with growth stages. In red flowered buckwheat (**Table 2**, Gan-Chao), cyanidin 3-*O*-galactosyl-rhamnoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, and cyanidin 3-*O*-rutinoside were detected at every growth stages, and cyanidin 3-*O*-rutinoside was the major anthocyanin. PAs–Cy content was also increased along with growth stages. From these results, it appears that the increase of fw and the synthesis/accumulation of each anthocyanins/PAs–Cy were synchronized with each other. To examine it statistically, we showed a correlation among the four anthocyanins contents, PAs–Cy contents, and fw (**Table 3**). In white (Kitawasesoba) and red (Gan-Chao) flowered buckwheat, each anthocyanins had significant positive correlations to fw and PAs–Cy contents. Therefore, to obtain anthocyanin-rich petals for food processing materials, the fully developed flower is suitable. In red flowered buckwheat, each anthocyanin also had a significant positive correlation to other anthocyanins except for the correlation of cyanidin 3-*O*-rutinoside to cyanidin 3-*O*-galactosyl-rhamnoside and to cyanidin 3-*O*-glucoside. This result indicates that cyanidin 3-*O*-

Table 2. Differences of Anthocyanin Contents in Red/White Flowers at Different Growth Stages[a]

	growth stage	mg fw/flower	cyanidin 3-*O*-galactosyl-rhamnoside (μg/petals in a flower)	cyanidin 3-*O*-glucoside (μg/petals in a flower)	cyanidin 3-*O*-rutinoside (μg/petals in a flower)	cyanidin 3-*O*-rhamnoside (μg/petals in a flower)	PAs–Cy (μg/petals in a flower)
kitawasesoba	1	0.37 ± 0.07	ND	ND	ND	ND	0.7 ± 0.5
	2	0.80 ± 0.03	ND	trace	trace	ND	6.1 ± 2.3
	3	1.40 ± 0.21	ND	trace	trace	ND	15.1 ± 0.8
	4	2.58 ± 0.19	ND	trace	0.04 ± 0.03	ND	27.9 ± 2.0
	5	3.42 ± 0.17	ND	trace	0.05 ± 0.01	ND	34.8 ± 2.1
Gan-Chao	1	0.28 ± 0.03	trace	0.07 ± 0.04	0.59 ± 0.28	0.03 ± 0.01	1.8 ± 0.8
	2	0.51 ± 0.04	trace	0.10 ± 0.04	0.72 ± 0.15	0.06 ± 0.03	4.1 ± 0.4
	3	0.99 ± 0.03	0.01 ± 0.01	0.15 ± 0.04	1.50 ± 0.18	0.16 ± 0.07	10.5 ± 3.3
	4	2.15 ± 0.12	0.14 ± 0.05	0.55 ± 0.08	2.64 ± 0.30	0.18 ± 0.08	13.9 ± 2.4
	5	3.13 ± 0.12	0.14 ± 0.06	0.57 ± 0.12	2.99 ± 0.12	0.30 ± 0.08	14.8 ± 3.1

[a] ND: not detectable (mean ± SD, $n = 3$).

Figure 2. Picture of buckwheat flowers in different growth stages. The pictures of flowers corresponding to each growth stage in **Table 2** are shown. Petals were harvested for each growth stage, respectively, which were classified according to fw's as follows: "Stage 1", 0.0–0.4 mg; "Stage 2", 0.4–0.8 mg; "Stage 3", 0.8–1.6 mg; "Stage 4", 1.6–2.6 mg; "Stage 5", 2.6–3.6 mg.

Table 3. Correlation Matrix of Values for fw, Anthocyanins, and PAs–Cy Contents

		fw	cyanidin 3-*O*-galactosyl-rhamnoside	cyanidin 3-*O*-glucoside	cyanidin 3-*O*-rutinoside	cyanidin 3-*O*-rhamnoside	PAs–Cy
Kitawasesoba	fw						
	PAs–Cy	0.995[c]					
Gan-Chao	fw						
	cyanidin 3-*O*-galactopyranosyl-rhamnoside	0.873[a]					
	cyanidin 3-*O*-glucoside	0.961[b]	0.996[c]				
	cyanidin 3-*O*-rutinoside	0.978[b]	0.966[b]	0.975[b]			
	cyanidin 3-*O*-rhamnoside	0.954[a]	0.586	0.861	0.944[a]		
	PAs–Cy	0.915[a]	0.969[b]	0.897[a]	0.966[b]	0.946[a]	

[a–c] Significant at (a) 5%, (b) 1%, and (c) 0.1% level.

rutinoside may have a different accumulation mechanism from that of other anthocyanins. It also indicates that the anthocyanin concentrations at fw bases are approximately the same among the different growth stages. In buckwheat leaf, rutin, which is the major flavonoid of the leaf, accumulates in the unexpanding young leaf to a very high concentration (about 20% of dry weight), and the concentration of rutin decreased along with the increase of leaf growth stages (*14*). From these results, anthocyanin and PAs–Cy in buckwheat flower had an accumulation mechanism different from that in its leaves.

From these results, we conclude that the fully developed petals of red flowered buckwheat, especially Gan-Chao, are promising as a new anthocyanin-rich material for food processing. Further studies that investigate the functional characterization of anthocyanins/PAs–Cy in the petals of red flowered buckwheat are required.

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