JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Identification of Anthocyanins in the Sprouts of Buckwheat

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The anthocyanin profiles and varieties/breeding line differences of anthocyanin concentrations in common/tartary buckwheat sprouts have been studied. Four anthocyanins, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-galactoside, and cyanidin 3-*O*-galactopyranosyl-rhamnoside, were isolated from the sprouts of common buckwheat, were separated using high-performance liquid chromatography (HPLC), and were identified using reversed-phase liquid chromatography (LC)/electrospray ionization−mass spectrometry (ESI-MS)/MS techniques. In tartary buckwheat sprouts, two anthocyanins (cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside) were identified. Among 19 common/tartary buckwheat varieties/breeding lines, Hokkai T10 contained the highest amounts of anthocyanins. Cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside concentrations in 6−10 days after seeding sprouts of Hokkai T10 ranged from 0.16 to 0.20 mg/g dry wt and from 5.55 to 6.57 mg/g dry wt, respectively. In addition, dark-grown sprouts of Hokkai T10 accumulated 0.091 and 2.77 mg/g dry wt of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside whereas other varieties/breeding lines accumulated trace amounts of anthocyanins. Given its anthocyanin-rich red cotyledons, Hokkai T10 is a promising line for use as "Moyashi" type sprouts and is strongly recommended as a new functional food, rich in dietary anthocyanins.

**KEYWORDS: Anthocyanins; ESI-MS; flavonoids; seed sprouts; buckwheat; light**

## INTRODUCTION

Anthocyanins are intensely colored water-soluble pigments, responsible for red, purple, or blue color attributes important in attracting consumers to flowers, fruits, and leaves. These compounds can be present in vegetative tissues, leaves, stems, flowers, roots, and emergent seedlings (*1*). Naturally occurring anthocyanins are composed of six aglycones (anthocyanidins) generally linked to sugar residues at the 3- or 5-hydroxy positions. They are hydrophilic and are generally present in plant cell vacuoles (*1*). Several recent reports have suggested that moderate consumption of anthocyanins is associated with a lowered risk of coronary heart disease and improvement of visual functions. The health benefits of anthocyanins are mainly attributable to their antioxidant activity, anticancer properties, and visual acuity enhancement (*2*). In common buckwheat sprout, the presence of radical-scavenging activity was demonstrated (*3*). In addition, change in DPPH-radical-scavenging activity was similar to that of the total flavonoid contents (*4*).

In recent years, seed sprouts, an atypical vegetable, have received attention as functional vegetables because of their beneficial nutritive value, including amino acids, fiber, minerals, and protein (*5*, *6*). In the Japanese market, a great variety of different types of sprouts can be found including those of broccoli (*Brassica oleracea* L. var. *italica* Plenck.), common buckwheat (*Fagopyrum esculentum* Moench), kale (*B. oleracea* L. var. *encephala*), mung bean (*Phaseolus aureus* Rob.), red cabbage (*B. oleracea* L. var. *capitata* f. Rubra), and soybean [*Glycine max* (L.) Merr.]. However, compared to the sprouts of common buckwheat, those of tartary buckwheat (*F. tataricum* Gaertn.) have recently received greater attention as a functional food given their 2.2-fold greater content of rutin, a flavonol glycoside known to strengthen blood vessels (*7*). In the Japanese market, there are presently available many kinds of buckwheat foodstuffs including alcohol, green sprouts, juices, and teas.

There have been few detailed reports regarding buckwheat seedling pigments and even fewer differentiating their levels among different plant organs such as flowers and stems.

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10.1021/jf0704716 CCC: $37.00 
Published on Web 06/20/2007

Watanabe and Shimizu (*3*) reported that the anthocyanin content of common buckwheat sprouts was increased from 0.25 to 1.80 mg/g dry wt by exposure to light for 3 days prior to harvest, but they did not identify the anthocyanins. In addition, anthocyanins, which are responsible for the red color of buckwheat sprouts, are very important because consumers in Japan prefer red-colored sprouts.

Recently, tartary buckwheat variety Hokkai T8 and Hokkai T10 have been developed by our research group. Interestingly, Hokkai T10, which was obtained through ethyl methane sulfonate mutagenesis of Hokkai T8, has a red hypocotyl and cotyledons during early sprout development stages, probably as the result of containing some anthocyanins. Therefore, the identification of individual anthocyanins and a time course of their levels during sprout growth were undertaken with these two tartary buckwheat lines. In Korea, buckwheat sprouts are consumed as "*Namul*" sprouts, grown in darkness in wooden jars like alfalfa, mung beans, and soybeans, because Korean consumers presumably prefer the mild flavor, slightly crisp texture, and very attractive fragrance of such sprouts (*8*). We therefore also investigated the effects of light on anthocyanin accumulation in Hokkai T8 and Hokkai T10 tartary buckwheat lines.

## MATERIALS AND METHODS

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

**Preparation of Plant Materials.** *Time Course Study of Anthocyanins.* Hokkai T8 and Hokkai T10 tartary buckwheat varieties were tested. Seeds were soaked in 10% (v/v) sodium hypochlorite (NaClO) for 3 h, were rinsed with Milli Q water for 1 h, and then were kept for 3 h at room temperature. Other materials employed in growing the sprouts were prewashed in 1% NaClO.

On September 2, 2005, pretreated tartary buckwheat seeds (8 g) were sown in plastic pots (65 × 65 × 150 mm) packed with polyurethane (Araikasei, Toyohashi, Japan). Twenty pots were prepared representing five different sprout age treatments (6−10 days after seeding; DAS), replicated four times. The seeds were germinated in a growth chamber for 2 days in the dark at 25 °C at roughly 60% humidity. Two DAS germinated seeds/pots were transferred to a greenhouse (mean temperature and relative humidity, 27 °C and 87%, respectively) at the National Agricultural and Food Research Organization for the Hokkaido region (Memuro, Hokkaido; longitude, 143°03′E; latitude, 42°55′N), where they were hourly sprayed with deionized water (mean pH 6.0) applied over a 5 min period. The growth benches were shaded with plastic netting, allowing roughly 5% of natural light to reach the developing sprouts (i.e., 16 and 504 $\mu$mol/m$^2$/s$^1$ irradiance inside and outside of the netting, respectively). Edible portions (shoot and cotyledons) of the sprouts were harvested, lyophilized, ground with an IFM-180G mill (Iwatani International Co., Tokyo, Japan), and stored in a sealed plastic bottle in a desiccator until chemical analysis.

*Effects of Light on Anthocyanin Accumulation.* Hokkai T8 and Hokkai T10 seeds, in three replicate batches, were sown at a seeding density of about 2.5 seeds/cm$^2$ (about 80 mg seed/cm$^2$) on sphagnum moss and then were covered with potting soil (*9*). The soil was removed at 4 DAS, and sprouts were thereafter grown with/without light (0 vs 13−14 $\mu$mol/m$^2$/s$^1$). The edible portion of the sprouts was harvested at 9 DAS, and their chlorophyll concentration was immediately measured by the method of Porra et al. (*10*). The remaining portion of samples was stored at −30 °C until they were used for measurement of anthocyanins.

*Varietal Differences of Anthocyanin Concentrations in Buckwheat Sprouts.* Ten common and nine tartary buckwheat varieties/breeding lines, in three replications, were sown and grown by the same method described above. Sprouts were harvested at 8 DAS (common buckwheat) and 9 DAS (tartary buckwheat) and then were stored at −30 °C until they were used for measurement of anthocyanins.

**HPLC/ESI-MS Analysis for Anthocyanins.** High-performance liquid chromatography (HPLC)/electrospray ionization−mass spectrometry (ESI-MS) analysis was carried out according to the method of Yamazaki et al. (*11*) with minor modifications. The anthocyanins were extracted overnight at 22 °C with 1 mL extraction solvent (MeOH/AcOH/$H_2O$, 80:0.2:19.8) per 8−10 mg dry wt of the sprouts. The extracts were passed through a 0.45 $\mu$m filter and were applied to an HPLC/ESI-MS system consisting of a Agilent 1100 Series LC (Agilent Technologies) coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). For the positive-ion ESI-MS performed at a capillary temperature of 365 °C and voltage of 3.5 kV, a nebulizing pressure of $N_2$ 50 psi was used as sheath gas. 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. Two HPLC methods were employed to separate anthocyanins: (1) to determine anthocyanin profiles, these compounds were separated using a 0−50 min linear gradient of 0−100% solvent A ($CH_3CN/H_2O$/TFA, 7.5:92.5:0.1) to solvent B ($CH_3CN/H_2O$/TFA, 55:45:0.1); (2) to compare to the anthocyanin profiles, anthocyanins were separated using an isocratic condition of 8% solvent A ($CH_3CN/H_2O$/TFA, 7.5:92.5:0.1) and 92% 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 (cyanidin 3-*O*-glucoside *m*/*z* 449, $[M + H]^+$; cyanidin 3-*O*-galactoside *m*/*z* 449, $[M + H]^+$; cyanidin 3-O-rutinoside, *m*/*z* 595, $[M + H]^+$; cyanidin 3-*O*-galactopyranosyl-rhamnoside, *m*/*z* 595, $[M + H]^+$) using a standard curve derived from commercial anthocyanins. As cyanidin 3-*O*-galactopyranosyl-rhamnoside was not commercially available, we used cyanidin 3-*O*-rutinoside instead.

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

**Statistical Analysis.** Analysis of variance and Tukey's multiple range test were applied using Esumi Statistical Software version 5.0 (Esumi Inc, Tokyo, Japan) and were used to determine statistically significant differences ($P \leq 0.05$).

## RESULTS AND DISCUSSION

**Separation and Structural Identification of Anthocyanins.** Some investigators have studied and quantified bulk buckwheat anthocyanins; however, given their lack of equipment to simultaneously separate and identify them, they failed to individually quantify them (*4*, *12*, *13*). The HPLC anthocyanin profiles of 8 DAS common buckwheat (cv. Gan-Chao) sprouts show three apparent anthocyanin peaks (**Figure 1A**). By comparison of their retention times and MS and MS/MS spectra to standards (**Figure 1D**), the three anthocyanins were identified as cyanidin 3-*O*-galactoside (*m*/*z* 449.3 ($[M + H]^+$)), cyanidin 3-*O*-glucoside (*m*/*z* 449.3 ($[M + H]^+$)), and cyanidin 3-*O*-rutinoside (*m*/*z* 595.3 ($[M + H]^+$)) (**Figure 1B** and **1C**). On the other hand, the extracted ion chromatogram (EIC) at *m*/*z* 595 gave two major peaks: cyanidin 3-*O*-rutinoside (**Figure 1C**) and an unknown with a similar retention time as peak A2 (**Figure 1A**). To identify it, this fraction was partially hydrolyzed, and its components were then compared to commercially obtained standards by HPLC and MS and MS/MS spectra (data not shown). Peak C1 (**Figure 1C**) was thus identified as cyanidin 3-*O*-galactopyranosyl-rhamnoside (*m*/*z* 595 ($[M + H]^+$)).

The sprouts of buckwheat thus contained only four anthocyanins, fewer than many other fruits or vegetables. For examples, berries (*14*) and blood oranges (*Citrus sinensis*) (*15*) contain more than 10 anthocyanins. One of the reasons for this

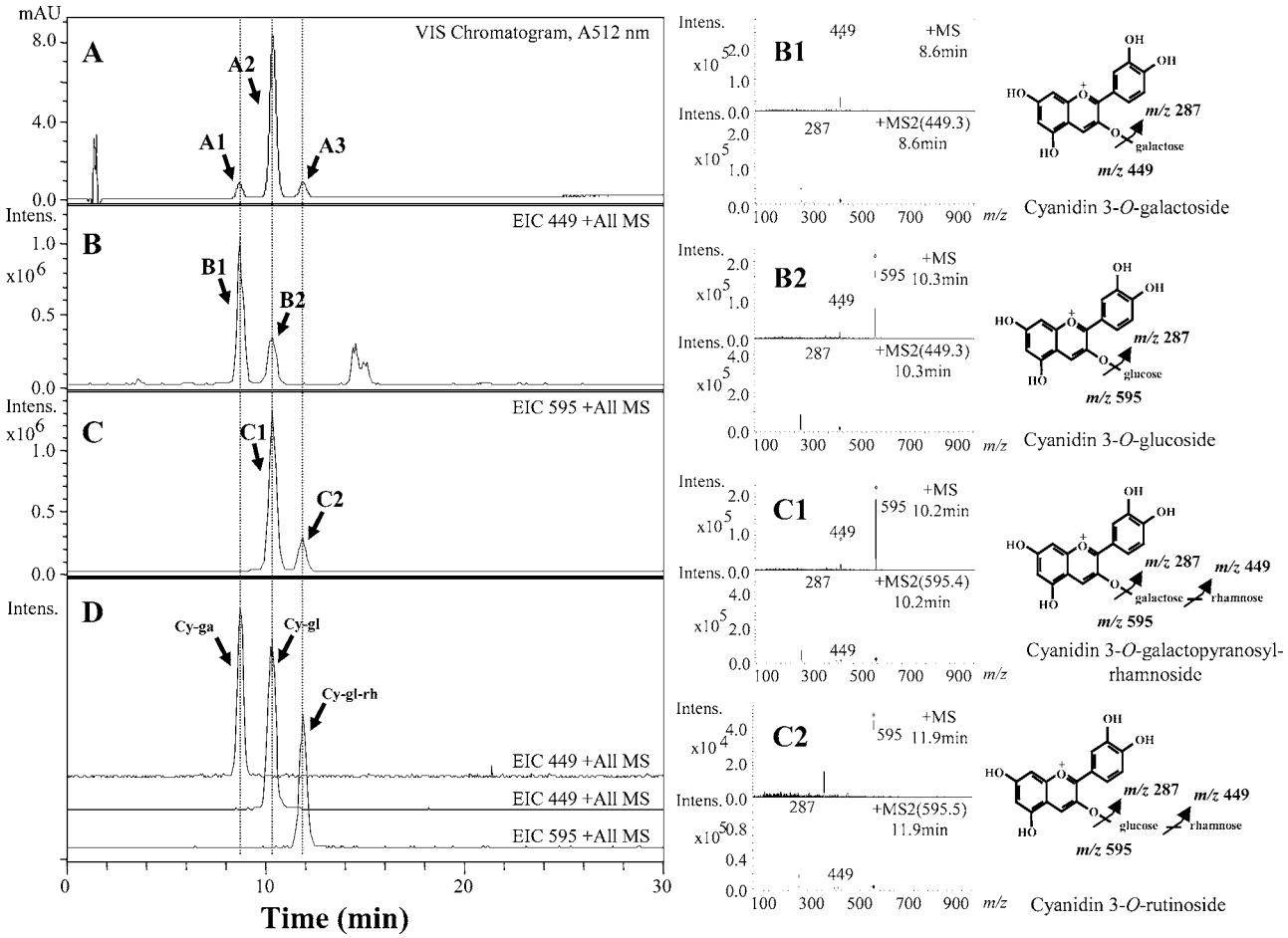


**Figure 1.** Identification of anthocyanins in common buckwheat sprouts (Gan-Chao). (**A**) Anthocyanin profile of petals of red-flowered buckwheat (detected at $A_{512}$). (**B**) EIC at *m*/*z* 449. (**C**) EIC at *m*/*z* 595. (**D**) Anthocyanin standards.

low diversity of anthocyanins in buckwheat is related to its single aglycone: in blueberry (*Vaccinium* spp.) (*14*), for example, anthocyanins bear over five different aglycones, including cyanidin and delphinidin, whereas cyanidin is the only aglycone present in tartary buckwheat sprouts.

Cyanidin 3-*O*-glucoside is the major anthocyanin of black soybean seed coats (*16*), and it is also found in some plants, such as berries (*14*), blood oranges (*Citrus sinensis*) (*15*), and purple corn (*Zea mays* L.) (*17*). It has many antioxidative and anti-inflammatory activities in vitro (*17*), and it prevents obesity and ameliorating hyperglycemia in mice (*18*). Cyanidin 3-*O*-rutinoside, found in blueberry fruit, exhibits inhibitory effects on the migration of human lung cancer cell lines (*19*). Therefore, anthocyanins identified in tartary buckwheat sprouts may be expected to have similar functions.

Rutinoside, the glycoside of cyanidin 3-*O*-rutinoside, is the same glycoside as in rutin, the major flavonol in buckwheat plants. This suggests that cyanidin glycosyltransferases and glycosidases in the cotyledons of tartary buckwheat probably have similar characteristics, in terms of sugar moiety substrate specificity, to those reported for flavonoid 3-*O*-glycosyltransferase and flavonol 3-*O*-glycosidase (*9*, *20*). Rutin and flavonol 3-*O*-glycosidase activity have been shown to be closely linked to the enhancement of the tartary buckwheat leaf stress defense system (*21*). Therefore, anthocyanins and their corresponding glycosidases may have similar roles in preventing the proliferation of microorganisms.

**Varietal Differences of Anthocyanins in Buckwheat Sprout.** To compare anthocyanin concentrations and compositions in common and tartary buckwheat sprouts, we investigated differences in the nature and level of anthocyanins in 10 common and 9 tartary buckwheat varieties/breeding lines (**Table 1**). In common buckwheat sprouts, we found four anthocyanins; cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-galactoside, and cyanidin 3-*O*-galactoside-rhamnoside. The most common anthocyanins in common buckwheat sprouts were cyanidin 3-*O*-galactoside-rhamnoside or cyanidin 3-*O*-galactoside, followed by cyanidin 3-*O*-rutinoside. On the other hand, we found only two anthocyanins in tartary buckwheat: cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside. Differences in numbers of compounds between common and tartary buckwheat sprouts were observed not only in anthocyanins but also in other flavonoids; tartary buckwheat sprouts contained rutin as a major flavonoid whereas common buckwheat sprouts contained many kinds of flavonoids such as rutin, orientin, isoorientin, vitexin, and isovitexin (*4*). As sources of red-colored sprouts, Gan-Chao, a Nepal native variety, and Hokkai T10 were promising because they contained high amounts of anthocyanins. Among them, Hokkai T10 contained a very high level of anthocyanins. Therefore, we performed time course analysis and investigated effects of light on anthocyanins accumulation, both important elements if one wishes to use Hokkai T10 as products of anthocyanin-rich sprouts.

**Time Course Analysis of Anthocyanins.** Tartary buckwheat sprouts reach a marketable size between 6 and 10 DAS, so knowing when anthocyanin content peaks can allow harvest to occur when the sprouts are at their nutritional prime in terms of these compounds. A time course of anthocyanin contents for

**Table 1.** Varietal Difference of Anthocyanin Concentrations in Buckwheat Sprouts

| name | cyanidin 3-*O*-glucoside mg/g.d.w. | cyanidin 3-*O*-rutinoside mg/g.d.w. | cyanidin 3-*O*-galactoside mg/g.d.w. | cyanidin 3-*O*-galactopyranosyl-rhamnoside mg/g.d.w. | origin |
|---|---|---|---|---|---|
| | | | Common Buckwheat | | |
| Kitawasesoba | trace | trace | 0.02 ± 0.01 | 0.10 ± 0.01 | Japan |
| Shinanonatsusoba | trace | trace | 0.17 ± 0.07 | 0.10 ± 0.02 | Japan |
| Hitachiakisoba | trace | trace | 0.07 ± 0.02 | 0.07 ± 0.01 | Japan |
| Nepal native | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.20 ± 0.02 | 0.16 ± 0.02 | Nepal |
| c.v. Monteneuf | trace | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.04 ± 0.00 | France |
| Heilongjiang Province native | trace | 0.01 ± 0.01 | 0.10 ± 0.02 | 0.06 ± 0.01 | China |
| Gan-Chao | trace | 0.01 ± 0.00 | 0.97 ± 0.09 | 0.18 ± 0.02 | China |
| Sumchanka | trace | trace | 0.08 ± 0.02 | 0.09 ± 0.01 | Russia |
| Skrosperaya 86 | trace | trace | 0.06 ± 0.00 | 0.06 ± 0.00 | Russia |
| | | | Tartary Buckwheat | | |
| Hokkai T8 | trace | 0.59 ± 0.05 | ND[a] | ND | Japan |
| Hokkai T10 | 0.13 ± 0.01 | 4.92 ± 0.32 | ND | ND | Japan |
| Ishisoba | trace | 0.50 ± 0.11 | ND | ND | Japan |
| Rotundatum | trace | 0.50 ± 0.10 | ND | ND | Russia |
| Tubrculatum | trace | 0.46 ± 0.12 | ND | ND | Russia |
| c.v. Pontivy | trace | 0.29 ± 0.04 | ND | ND | France |
| Putong-Kuqiao | trace | 0.40 ± 0.07 | ND | ND | China |
| Jianzui-Kuqiao | trace | 0.39 ± 0.07 | ND | ND | China |
| Tite Phapal | trace | 0.25 ± 0.05 | ND | ND | Nepal |
| Yugoslavia | trace | 0.44 ± 0.07 | ND | ND | Yugoslavia |

[a] ND: not determined because of undetectable amo (mean ± SD, $n = 3$).

**Table 2.** Anthocyanin Concentration on the Edible Parts in the Sprouts of Tartary Buckwheat[a]

| days after sowing | cyanidin 3-*O*-glucoside mg/g.d.w. | | cyanidin 3-*O*-rutinoside mg/g.d.w. | |
|---|---|---|---|---|
| | Hokkai T8 | Hokkai T10 | Hokkai T8 | Hokkai T10 |
| 6 | trace | 0.20 ± 0.01 a | 0.18 ± 0.01 a | 5.55 ± 0.16 b |
| 7 | trace | 0.18 ± 0.02 ab | 0.19 ± 0.01 a | 6.06 ± 0.28 ab |
| 8 | trace | 0.16 ± 0.01 b | 0.19 ± 0.01 a | 6.04 ± 0.22 ab |
| 9 | trace | 0.16 ± 0.02 b | 0.17 ± 0.01 a | 6.57 ± 0.47 a |
| 10 | trace | 0.17 ± 0.02 ab | 0.17 ± 0.02 a | 6.28 ± 0.32 a |

[a] Mean ± SD, $n = 4$. Values within the same row with different alphabet letters are significantly different ($p < 0.05$) among groups by Tukey's multiple range test.

6−10 DAS sprouts of each line (**Table 1**) shows that cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside concentrations in Hokkai T10 ranged from 0.16 to 0.20 mg/g dry wt and from 5.55 to 6.57 mg/g dry wt, respectively, whereas only trace amounts of either were detected in Hokkai T8. Thus, Hokkai T10 maintained high anthocyanin levels throughout the period, well in excess of those found in Hokkai T8. From these results, one can conclude that Hokkai T10 is superior to Hokkai T8 in terms of producing anthocyanin-rich sprouts.

**Effects of Light on Anthocyanin Accumulation.** Hokkai T8 and Hokkai T10 were grown with or without light to investigate effects of light on anthocyanin accumulation. Light-grown 9 DAS Hokkai T10 sprouts accumulated 0.13 and 4.92 mg/g dry wt, respectively, of cyanidin 3-*O*-glucoside (**Figure 2A**) and cyanidin 3-*O*-rutinoside (**Figure 2B**). On the other hand, non-chlorophyll-accumulating (**Figure 2C**) dark-grown sprouts can accumulate 0.09 and 2.77 mg/g dry wt of cyanidin 3-*O*-glucoside (**Figure 2A**) and cyanidin 3-*O*-rutinoside (**Figure 2B**), whereas Hokkai T8 accumulated only trace amounts of anthocyanins. In general, most dark-grown plants accumulated few anthocyanins compared to light-grown plants (*22*, *23*). Common and tartary buckwheat varieties/breeding lines in **Table 1** accumulated only a trace amount of the anthocyanins (data not shown), whereas dark-grown Hokkai T10 accumulated 56% of the cyanidin 3-*O*-rutinoside compared to light-grown condition. This indicates that Hokkai T10 may have a unique regulation mechanism for dark accumulation of anthocyanins

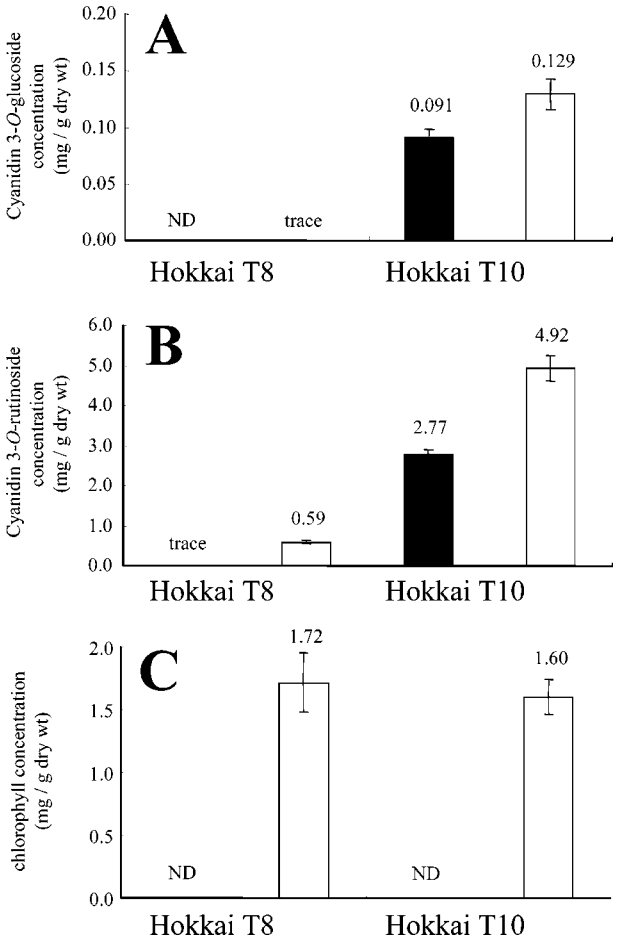


**Figure 2.** Effects of light on anthocyanin and chlorophyll concentrations. Black, in darkness; white, in light. (**A**) Cyanidin 3-*O*-glucoside concentration; (**B**) cyanidin 3-*O*-rutinoside concentration; (**C**) chlorophyll concentration. Data are means of three independent experiments. Bars indicate standard deviation. n.d.: not determined.

compared with other plants, since the hypocotyls and cotyledons of Hokkai T10 are deep red even in darkness. In Japan, most "Moyashi", a generic name of sprouts grown in darkness, are

of a yellowish color. Therefore, Hokkai T10 would be promising as colored sprouts including Moyashi.

The sprouts of Hokkai T10 may thus prove to be a useful anthocyanin-rich foodstuff. In Japan, in recent years, tartary buckwheat has been commercialized in such goods as seed (green) sprouts and lyophilized powder for alcohol, juice, and teas rich in rutin. Lyophilized powder is widely used mixed with other foods including bread or confectionery. Lyophilized powder of Hokkai T10 would constitute a high-quality additive given its deep-red color. In buckwheat, anthocyanins can be found all above ground organs including cotyledons, leaves, and stems. Variation in anthocyanin and vitamin content according to plant organs and growth stages of buckwheat would have important implications for human health.

## ACKNOWLEDGMENT

We thank Mr. A. Morizumi, Mr. S. Nakamura, and Mr. K. Suzuki for technical assistance.

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**Received for review February 17, 2007. Revised manuscript received May 4, 2007. Accepted May 7, 2007. This study was supported by the Northern Advancement Center for Science and Technology and Cooperation of Innovative Technology and Advanced Research in Evolutional Area (CITY AREA).**

JF0704716