Differences in the Ratios of Cyanidin-3-O-glucoside and Cyanidin-3-O-rutinoside to Total Anthocyanin under UV and Non-UV Conditions in Tartary Buckwheat (Fagopyrum tataricum Garten)

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Abstract: Anthocyanins play beneficial roles in plant growth and development such as the reduction of photo-oxidative damage to leaf cells. Tartary buckwheat contains two anthocyanins, namely, cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, which are accumulated in the stems and leaves. In order to clarify which type of anthocyanin is accumulated at different nodal positions, we investigated the type of anthocyanin and their contents in buckwheat stems using HPLC. The anthocyanins detected were identified as cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside by comparison with the retention times and co-chromatography with the standard solutions. The contents of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside at the proximal stem position were higher than those at the distal stem position. The ratio of each anthocyanin to total anthocyanin varied with the nodal positions. An outdoor study suggested that UV stress might influence the ratio of each anthocyanin to total anthocyanin. Consequently, we investigated these ratios in a growth chamber. The growth chamber study suggested that the ratio of cyanidin-3-O-rutinoside to total anthocyanin was higher under UV conditions than under non-UV conditions. These results indicate that cyanidin-3-O-glucoside accumulates in a small amount and cyanidin-3-O-rutinoside accumulates in a large amount in young organs that suffer from strong UV stress. Cyanidin-3-O-rutinoside may have a UV-protective effect and tartary buckwheat may accumulate cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside systematically to afford effective protection against UV stress.

Key words: Anthocyanin, Flavonoids, Node.

Anthocyanins belong to the flavonoid group of polyphenolic compounds responsible for the red and blue colors of plant organs such as fruits, flowers, and leaves (Strack and Wray, 1993). Anthocyanins play an important role in the prevention of oxidative damage caused by active oxygen radicals (Gabrielska et al., 1999). The biological functions of anthocyanins in plants have been widely discussed (Willson and Whelan, 1990; Gould and Lister, 2006), and they have been clarified to play various roles in plant growth and development such as reducing the risk of photo-oxidative damage to leaf cells by masking chlorophylls (Field et al., 2001; Hoch et al., 2003; Lee et al., 2003).

Tartary buckwheat (Fagopyrum tataricum Garten) is a cereal crop plant belonging to the Polygonaceae family (Campbell, 1997; Wijingaard and Arendt, 2006). The flour of tartary buckwheat is used to make noodles, and in some countries the young parts of the plant are consumed as a vegetable. Tartary buckwheat is recognized as a healthy food since it contains flavonoids in its seeds, stems, and leaves. Anthocyanins are formed throughout the entire growth term in the stems of tartary buckwheat. The red hypocotyls of buckwheat are said to be produced by the accumulation of cyanidin glycoside (Troyer, 1958, 1964). Kim et al. (2007) reported the isolation of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside from the hypocotyls of tartary buckwheat.

The extent of environmental stress experienced by a plant is assumed to differ with the nodal positions. In this study, we examined the type and quantity of anthocyanin accumulated in the stems and hypocotyls of tartary buckwheat, and investigated whether the type of anthocyanin vary with the nodal position under outdoor conditions. In order to investigate the relationship between the types of anthocyanin and UV radiation, we grew tartary buckwheat hypocotyls under UV and non-UV conditions in two growth chambers.

Materials and Methods
1. Plant growth under outdoor conditions

Seeds of the tartary buckwheat cultivar "Rotundatum" were sown on April 11, 2008 in each plot arranged in a randomized complete block design with 4 replications in a field of the National Agricultural Research Center for Kyushu Okinawa Region (Kumamoto, Japan). The plot size was 900 cm², and the seeding density was 125 plants m⁻². We sampled the young adult plants (22 days after sowing) from each plot, when the youngest node (4th node from the surface of the soil) had expanded.
The main stem of each plant was divided into 3 parts: the proximal-node (1st node), middle-node (2nd node), and distal-node (3rd node).

2. Plant materials and growth chamber conditions
Seeds of the tartary buckwheat cultivars “Rotundatum,” “Nepal,” and “Yugoslavia” were sown in plastic pots (50 × 35 × 8 cm, length × width × height) containing commercial garden soil (Akadamatsuchi, Ohshi Co., Japan) in two growth chambers (Nippon Medical & Chemical Instruments Co., Japan). The growth chambers were controlled at 15ºC, relative humidity of ca. 70%, under continuous irradiation. Seeds were sown at a spacing of 1.0 cm (1 plant cm⁻²) at a sowing depth of 5 mm. In this experiment, each variety was arranged in a randomized complete block design with 4 replications. The plot size for each variety was 36 cm². Irrigation was conducted several times a day. The two light treatments were UV and non-UV conditions. Light intensity was recorded on the surface of the soil. For the non-UV light condition, three white fluorescent lamps (FL20SS·D/18, Matsusihita Co., Japan) were placed parallel to each other. The light intensity was adjusted to 74 μmol m⁻² s⁻¹ (TMS220; Tasco Co., Japan) and the UV intensity was 0.0 mW m⁻² (UV-MONI; Archiweb Co., Japan). For the UV light condition, three UV lamps (FLS20S·BL-B; Matsusihita Co., Japan) were mounted between three white fluorescent lamps at regular intervals, and the fluorescent light and UV intensities were adjusted to 74 μmol m⁻² s⁻¹ and 30.0 mW m⁻², respectively. Hypocotyls were harvested at 8, 11, 17 and 25 days after sowing.

3. Extraction of anthocyanin
Fresh samples were frozen with liquid nitrogen and then freeze-dried using a freeze dryer (model FDU-1200; EYELA, Japan). Dried samples were pulverized using beads (Bead Smash 12; Waken, Japan). Anthocyanin was extracted from powdered samples with methanol-concentrated HCl (99/1, v/v) for 24 hr at 4°C. The sample was then centrifuged at 10,700 × g for 10 min. After collecting the supernatant, methanol-concentrated HCl (99/1, v/v) was added to the residue and re-centrifuged as described above. The two supernatants were mixed and subsequently purified using a membrane filter (0.45 μm; Whatman plc., USA) prior to anthocyanin analysis by high-performance liquid chromatography (HPLC).

4. Identification of anthocyanins using HPLC
HPLC analysis was performed according to Watanabe and Ito (2003) with certain modifications. The sample solution (20 μL) was injected into an HPLC system (LaChrom Elite; Hitachi Ltd., Japan). HPLC was conducted using a Cadenza CD-C18 column (3 μm, 250 × 4.6 mm i.d.; Intakt Corp., Kyoto, Japan) at 35°C. The elution system consisted of mobile phase (A) of water/methanol/acetic acid (92.5:5.0:2.5, v/v/v) and mobile phase (B) of water/methanol/acetic acid (2.5:95.0:2.5, v/v/v). Elution was performed using a linear gradient of B into A at a flow rate of 0.75 mL min⁻¹; elution starting with 5% B; 0–60 min, 5–40% B. A chromatograph was monitored at 520 nm and the anthocyanin concentrations were determined by comparison with a standard curve derived from commercial cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside (Funakoshi Co. Ltd., Tokyo, Japan).

Results
1. Anthocyanins in the stems of tartary buckwheat
The HPLC profile of the acidified methanolic extract from the hypocotyls of tartary buckwheat “Rotundatum” exhibited two apparent anthocyanin peaks (Fig. 1). By comparing with the retention times and co-chromatography with the standard solutions, peaks 1 and 2 were identified as cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside (Funakoshi Co. Ltd., Tokyo, Japan).

![Fig. 1. Anthocyanins in the hypocotyls of the cultivar Rotundatum of tartary buckwheat. Peak 1, cyanidin-3-O-glucoside; Peak 2, cyanidin-3-O-rutinoside.](image)

| Positions | number | cyanidin-3-O-glucoside (mg (g Dry Weight)⁻¹) | cyanidin-3-O-rutinoside (mg (g Dry Weight)⁻¹) | total anthocyanin (mg (g Dry Weight)⁻¹) |
|-----------|--------|------------------------------------------|-------------------------------------------|-----------------------------------|
| Distal    | 3      | 0.014 ± 0.003 c                          | 0.362 ± 0.067 c                          | 0.376 ± 0.070 c                   |
| Middle    | 2      | 0.032 ± 0.005 b                          | 0.682 ± 0.121 b                          | 0.715 ± 0.126 b                   |
| Proximal  | 1      | 0.076 ± 0.005 a                          | 1.054 ± 0.082 a                          | 1.130 ± 0.084 a                   |

The data shown are the means of 4 replications ±SD, and were analyzed using a one-way ANOVA followed by multiple comparison tests using Fisher’s least significant difference (LSD) test. Different letters (a, b, and c) indicate a significant difference (P < 0.01).
and cyanidin-3-β-rutinoside, respectively. The retention times of peaks 1 and 2 were 26.5 and 29.6 min, respectively.

2. Contents of each anthocyanin in the stems of tartary buckwheat

In order to clarify the composition of the two anthocyanins in the stems, we investigated the contents and the ratios of each anthocyanin to total anthocyanin in the stems at different nodal positions. Table 1 shows that the contents of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside decrease concomitantly with an increase in the height of the stem position in plants at the young adult stage. The content of cyanidin-3-O-rutinoside in the proximal position was markedly higher than that in the distal and middle positions. In all stem positions, the amount of cyanidin-3-O-glucoside was considerably smaller than that of cyanidin-3-O-rutinoside.

The ratio of cyanidin-3-O-rutinoside to total anthocyanin in the distal position was higher than that in the proximal position. The ratio of cyanidin-3-O-glucoside to total anthocyanin decreased concomitantly with an increase in the height of the stem position (Fig. 2).

3. The contents of each anthocyanin in the hypocotyls of tartary buckwheat under UV and non-UV light conditions

The impact of UV light on anthocyanin production was evaluated with tartary buckwheat hypocotyls in growth chambers. From the average of “Rotundatum,” “Nepal,” and “Yugoslavia,” the content of cyanidin-3-O-rutinoside was 2.3 times higher under UV conditions than under non-UV conditions at 25 days after sowing (Fig. 3 D). The content of cyanidin-3-O-glucoside was 2.0 times higher under UV conditions than under non-UV conditions at 25 days after sowing (Fig. 4 D).
Fig. 4. The contents of cyanidin-3-O-glucoside at different sampling times. (A) Yugoslavia (n=4); (B) Rotundatum (n=4); (C) Nepal (n=4); (D) Average of the 3 varieties (n=12). The data were analyzed using ANOVA. ** and * indicate significance at the 1% and 5% levels, respectively. n.s. indicates non-significance. The vertical bar on each spot indicates the SD.

Fig. 5. The ratio of cyanidin-3-0-rutinoside to total anthocyanin at different sampling times under UV and non-UV conditions. (A) Yugoslavia (n=4); (B) Rotundatum (n=4); (C) Nepal (n=4); (D) Average of the 3 varieties (n=12). The data were analyzed using ANOVA. ** and * indicate significance at the 1% and 5% levels, respectively. n.s. indicates non-significance. The vertical bar on each spot indicates the SD.
These results showed that the contents of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside increase under both UV and non-UV condition, as the hypocotyls grow.

From the average of “Rotundatum,” “Nepal,” and “Yugoslavia,” the effect of UV radiation on the ratio of cyanidin-3-O-rutinoside to total anthocyanin was analyzed statistically (Fig. 5). A significant difference (1% level) in the ratio was observed between UV and non-UV conditions at 8, 11, and 17 days after sowing. In addition, the same tendency was confirmed at 25 days after sowing. Thus, under UV light conditions, the ratio of cyanidin-3-O-rutinoside to total anthocyanin was higher than that under the non-UV conditions (Fig. 5D). In contrast, the ratio of cyanidin-3-O-glucoside to total anthocyanin under the UV conditions was lower than that under the non-UV condition.

**Discussion**

In this study, two types of anthocyanin were found in the stems and hypocotyls of tartary buckwheat; these were identified as cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside. The identities of these two anthocyanins are consistent with those described in a previous report (Kim et al., 2007). We observed that the ratio of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside to total anthocyanin varied with the nodal position.

Tartary buckwheat may accumulate anthocyanins to protect from stresses such as UV radiation. Beckwith et al. (2004) reported that the types and ratios of anthocyanins in *Pennisetum setaceum* were altered under high-intensity light conditions. Similar to the situation in *P. setaceum*, the anthocyanins in tartary buckwheat might be converted into other anthocyanins as a consequence of environmental damage. Thus, the ratio of anthocyanins is expected to differ between the upper and proximal nodal position, the former receiving more solar radiation than the latter. Our results indicate that in tartary buckwheat the ratio of cyanidin-3-O-glucoside to total anthocyanin was decreased in the upper nodal position compared with lower nodal position.

The antioxidant capacity of cyanidin-3-O-rutinoside is slightly higher than that of cyanidin-3-O-glucoside (Heo et al., 2007), or almost equivalent (Lichtenthaler et al., 2003). Our results suggested that tartary buckwheat might increase its antioxidant capacity by generating cyanidin-3-O-rutinoside at a high ratio to total anthocyanins in the upper nodal position which is exposed to excess UV stress. Rubinskiene et al. (2005) reported that cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside occur in blackcurrant and that cyanidin-3-O-rutinoside is more stable against thermal treatment than cyanidin-3-O-glucoside. Reen et al. (2006) reported that N-nitrosomethylbenzylamine metabolism from cyanidin-3-O-rutinoside is strongly inhibited by cyanidin-3-O-glucoside. Although the investigators (Rubinskiene et al., 2005; Reen et al., 2006) did not mention the protective effect of anthocyanins in plant tissues, their results suggest that the type of sugar residues of anthocyanidins is important for physiological functions. The results of the present study clarified that tartary buckwheat accumulates cyanidin-3-O-rutinoside and cyanidin-3-O-glucoside at different ratios at different nodal positions. All these findings suggest that each type of anthocyanin in the stems of tartary buckwheat has a different physiological function.

The anthocyanin contents of plants grown in growth chambers were higher than those of plants grown outdoors. Watanabe and Ito (2003) reported that continuous light treatment promoted anthocyanin synthesis and inhibited extension growth of buckwheat seedlings. Miura et al. (1983) reported that anthocyanin contents increased at lower temperatures in seedlings of benitade (*Polygonum hydropiper* L.). The growth chamber conditions used in the present study (24/0 h light/dark photoperiod, 15°C) were conducive to the elevation of anthocyanin contents. Under these growth chamber conditions, we demonstrated that the contents of anthocyanin in tartary buckwheat hypocotyls increase with growth, and that UV light increases the anthocyanin content. Our results are consistent with those obtained by Watanabe and Ito (2003). We revealed that the ratio of cyanidin-3-O-rutinoside and that of cyanidin-3-O-glucoside to total anthocyanin was higher and lower, respectively, under UV conditions than under non-UV conditions, and that the ratio of cyanidin-3-O-glucoside to total anthocyanin was lower under UV conditions than under non-UV conditions. The expression of anthocyanin biosynthetic genes has been demonstrated to be enhanced by UV in apple (Ubi et al., 2006). UV radiation may increase glycosyltransferase activity, thus facilitating the conversion of the glucoside attached to cyanidin to rutinoside. The present study was conducted both outdoors and in growth chambers to demonstrate that tartary buckwheat can accumulate appropriate types of anthocyanin to protect plant tissue against UV radiation.

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