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Anthocyanins in perilla plants and dried leaves

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ABSTRACT

High-quality perilla leaves are purple on upper and lower surfaces and have a good aroma. The Japanese Pharmacopoeia specifies the content of essential oils in perilla leaves but not that of anthocyanins. Several reports have described the chemical species of anthocyanins in red perilla, but a complete analysis of anthocyanins in perilla has not been reported. In this study, the anthocyanins in the leaves of cultivated and wild species of perilla and those in commercially available perilla herbs were studied. Red perilla and most *P. citriodora* strains accumulate cyanidin derivatives that differ in the acyl group on the glucose moiety at the 3-*O*- and 5-*O*-positions of the anthocyanins. Several strains of *P. citriodora* contain cyanidin derivatives that are different from those in red perilla and most *P. citriodora* species. Green perilla and wild species other than *P. citriodora* do not contain foliar anthocyanins. The anthocyanins in commercially available perilla herbs and natural dyes made from red perilla were in agreement with those in fresh red perilla leaves and most *P. citriodora* samples. The amounts and types of anthocyanins were not associated with place of cultivation, although some changes occurred due to degradation during storage. These results provide clues regarding the biosynthesis of anthocyanins in perilla and the evolution of red perilla. The characteristics and stability of anthocyanins are discussed.

1. Introduction

Perilla frutescens is an annual plant used as a fresh herb in cooking. Purple perilla leaves are used pharmaceutically to ease stomach disorders and induce sweating (Ministry of Health, Labour and Welfare, 2016), and are blended in several Japanese traditional medicinal formulations such as Kososan, Shimpito, and Hangekobokuto. According to *Honzokomoku*, an ancient Chinese textbook of medicinal plants, high-quality perilla leaves are purple on both sides and have a good aroma (Shirai, 1930).

The reddish color of perilla leaves is due to anthocyanin pigments. Anthocyanins have antioxidant activity, and natural materials containing these compounds are often used as ingredients in health foods. Anthocyanins are also used as natural food coloring, which most consumers prefer to synthetic food coloring. Specifically, anthocyanins and other dyes of plant origin are used to impart red, purple, or blue colors to food. Perilla has traditionally been used to color pickles and drinks in Japan, and food dyes made from red perilla have recently become available as commercial products. However, anthocyanins in perilla leaves are unstable and are easily damaged by high pH, light, heat, and ionized compounds (Goto et al., 1976; Yoshida et al., 1990). Several studies have identified some of the anthocyanins in red perilla, but a comprehensive analysis has not been reported.

In Japan, there are four *Perilla* species: a cultivated species, *P. frutescens*, and three wild species, *P. citriodora*, *P. hirtella*, and *P. setoyensis*. Each has unique characteristics and the number of chromosomes differs between the cultivated species ($2n = 40$) and the wild species ($2n = 20$). The results of our previous experiments on genetic crossing and DNA polymorphisms suggest that *P. frutescens* is an amphidiploid of two wild species and that *P. citriodora* may be one of the original species (Ito et al., 1998). *P. citriodora* leaves are red on the abaxial side but not on the adaxial side. It would be interesting to elucidate the mechanism controlling the red color of perilla leaves. In this study, the anthocyanins in the leaves of cultivated and wild species of perilla, as well as those in commercially available perilla herbs, were identified. In addition, a possible biosynthetic pathway for anthocyanins is postulated from the chemical species of anthocyanins, and the chemotaxonomical implications of our findings are discussed.

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2. Results and discussion

2.1. HPLC analysis of anthocyanins in fresh and dried perilla leaves and in natural dyes made from perilla

The HPLC patterns of samples of different perilla strains were different and could be divided into four groups (Fig. 1). A maximum of seven peaks are present in each HPLC pattern. All red perilla samples (*P. frutescens*), most *P. citriodora* samples, and commercially available perilla herbs and natural dyes made from red perilla provided pattern A (five peaks, Nos. 3 to 7). Only one strain of *P. citriodora* (Strain No. 87) showed pattern B (seven peaks, Nos. 1 to 7), and another strain of *P. citriodora* (Strain No. 5717) showed pattern C (two peaks, Nos. 1 and 2). In contrast, most green perilla (*P. frutescens*) samples, as well as *P. setoyensis* and *P. hirtella* samples, provided no anthocyanin peaks (pattern D).

2.2. Identification of anthocyanins

Anthocyanins in perilla could be identified by comparing their retention times with those of authentic compounds; however, some of the authentic anthocyanins were not available. The MS and NMR spectra of these anthocyanins were examined to determine their aglycone structures. Anthocyanidins found in plant anthocyanins are classified as pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, and they have different patterns of hydroxyl or methoxy groups on the B-ring. The more hydroxyl groups an anthocyanidin has, the bluer it appears. Pelargonidin with one hydroxyl group is orange and has a maximum absorption at 515 nm; cyanidin with two hydroxyl groups is reddish violet and has a maximum absorption at 528 nm; and delphinidin with three hydroxyl groups is blue-violet and has a maximum absorption at 543 nm (Yokoi et al., 1979). Previous reports on perilla anthocyanidins showed that almost all the aglycones were cyanidin (Yoshida et al., 1990; Yamazaki et al., 2003), and cDNA clones encoding the enzymes involved in anthocyanin biosynthesis, namely, chalcone synthase, flavanone 3-hydroylase, dihydroflavonol 4-reductase, UDP glucose: flavonoid 3-*O*-glucosyltransferase, and flavonoid 3′-hydroxylase have been isolated from the leaves of a red perilla (Gong et al., 1997; Kitada et al., 2001). Therefore, the seven anthocyanidins found in perilla were expected to have cyanidin as an aglycone, and this was supported by the maximum absorption of 528 nm for the seven compounds. The compounds were further analyzed by MS and NMR to confirm their structures. Anthocyanins were extracted from red leaves of Strain No. 5717 (73.7 g) and Strain No. 5601 (34.5 g) and purified to single compounds. The yields of the compounds based on HPLC peak areas were as follows: peak 1, 32.1 mg; peak 2, 12.7 mg; peak 3, 13.7 mg; peak 4, 4.6 mg; peak 5, 5.8 mg; peak 6, 8.6 mg; and peak 7, 38.2 mg. Peaks 1 and 2 were purified from Strain No. 5717 and peaks 3, 4, 5, 6, and 7 were purified from Strain No. 5601. The compounds corresponding to peaks 1, 3, 4, 5, 6, and 7 were identified by MS and ^{1}H NMR analyses and are shown in Fig. 2 (Charron et al., 2007; He et al., 2010; Kondo et al., 1989; Yoshida et al., 1990). These compounds are cyanidin derivatives with different acyl groups on the glucose moiety attached to the anthocyanin 3-*O*- and 5-*O*-positions. The MS data for peak 3 showed the co-existence of two compounds that were subsequently separated. The ^{1}H NMR data of peak 2 did not correspond to any previously reported anthocyanin and MS showed peaks at *m/z* 697.16 $[M]^{+}$, 535.11 [cyanidin + glucose + malonyl]$^{+}$, 449.11 $[Cy + Glc]^{+}$, and 287.05 $[Cy]^{+}$. Perilla expresses enzymes that catalyze malonylation at the 6′-*O*-position of 5-*O*-glucose (Matsune et al., 1997; Suzuki et al., 2001). The ^{1}H NMR data of peak 2 were similar to those of cyanidin-3,5-*O*-diglucoside (Yoshida et al., 1990), but a proton signal corresponding to the 6′-C-position in glucose A was shifted downfield (data reported by Goto et al. (1976): 3.96, 3.75 → data of peak 2: 4.55, 4.34). This shift can be explained by the malonylation of glucose A. Other peak 2 signals shifted upfield and corresponding to glucose were similar to pelargonidin 3-*O*-glucoside-5-*O*-malonylglucoside, a compound malonylated at glucose B of pelargonidin 3,5-*O*-diglucoside (Hosokawa et al., 1995). Peak 2 was thus identified as cyanidin 3-*O*-glucoside-5-*O*-malonylglucoside. The ^{13}C NMR and ^{1}H NMR signals of peak 2 were assigned using 2D NMR and are shown in Table 3.

Previous studies on perilla anthocyanins have had conflicting results regarding the compounds present in red perilla leaves. For example, Yoshida et al. (1990) found compounds 1 and 3 to 7 and Yamazaki et al. (2003) found compounds 2, 3a, and 4 to 7. No previous report is consistent with the compounds in the present report (compounds 3 to 7). This discrepancy is in part due to the degradation of anthocyanins: compound 1 in Yoshida's report and compound 2 in Yamazaki's report could be formed by the degradation of compounds 3 to 7 during extraction and storage prior to analysis. Compound 3b is absent from Yamazaki et al.'s report because they only used HPLC-PDA-MS for compound identification and thus could not separate compound 3b from its isomer, compound 6.

In summary, red perilla and most *P. citriodora* stains accumulate compounds 3, 4, 5, 6, and 7, and two strains of *P. citriodora* (Strain Nos. 87 and 5717) produce the anthocyanins compounds 1 and 2. Green perilla and wild species of perilla other than *P. citriodora* do not contain foliar anthocyanins.

2.3. Oil type and anthocyanin pattern are genetically independent

Table 1 shows the anthocyanin pattern of fresh perilla leaves corresponding to Fig. 1 and their oil type. Perilla oils can be

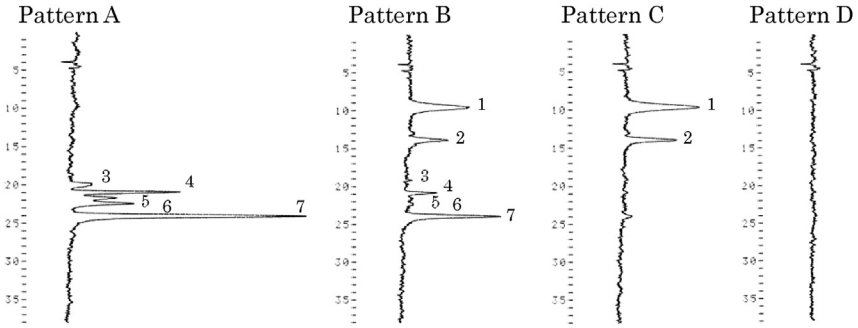

Fig. 1. HPLC chromatograms of perilla anthocyanins.