赤花ペチュニア品種における微量アントシアニンの同定

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Determination of Minor Floral Anthocyanins in a Red-flowered Petunia

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Summary

Minor floral anthocyanins of a red-flowered petunia were isolated and determined by chromatographic and spectroscopic methods. Petunidin 3-glucoside (1.7%) and malvidin 3-glucoside (1.3%) were determined for the first time in the genus *Petunia*. Incomplete substrate specificity of anthocyanin 3'- and 3', 5'-methyltransferase is considered to be a cause for accumulating a small amount of 3-glucosides of petunidin and malvidin in the flowers.

Key Words: anthocyanin, malvidin 3-glucoside, petunidin 3-glucoside, red-flowered petunia, Solanaceae.

Introduction

Petunia × *hybrida* Vilm. (petunia) is a model plant of the biosynthetic pathway of anthocyanin pigments (Cornu and Maizonnier, 1983; Gerats and Martin, 1992; Holton and Cornish, 1995; Tanaka et al., 1998; Wiering and de Vlaming, 1984). To our knowledge, 40 different anthocyanins have been unambiguously determined from flowers of wild species of *Petunia* (Ando et al., 1999; Tatsuzawa et al., 1997, 1999, 2000) and cultivars (Ando et al., 2000; Fukui et al., 1998; Gonzalez et al., 2001; Slimestad et al., 1999; Tatsuzawa et al., 2004), The most complex is di-acylated malvidin 3-rutinoside-5-glucoside (Fukui et al., 1998; Gonzalez et al., 2001; Tatsuzawa et al., 1997), whereas the simplest one is cyanidin 3-glucoside (Ando et al., 2000). However, many other minor anthocyanins that constitutively contribute to flower color are still unidentified. For example, Ando et al. (2000) surveyed floral anthocyanins in 28 commercial red flowered petunias and found cyanidin 3-sophoroside, cyanidin 3-glucoside, and peonidin 3-glucoside as the major components, but several minor anthocyanins were not identified. In this paper, we report the isolation and identification of three of these minor anthocyanins in red petunia 'Baccara Red'.

Materials and Methods

Plant materials

A commercial red petunia 'Baccara Red' (Sakata Seed Co., Ltd, Yokohama) was raised from seed in May, 2004 and grown in a greenhouse by following standard floricultural practice.

HPLC analysis of anthocyanins from fresh flowers

Fresh corolla-limbs (ca. 0.02 g) of 'Baccara Red' were extracted with 1 mL MAW (methanol (MeOH)-acetic acid (HOAc)-water (H_2O), 9:1:10, v/v/v), according to Ando et al. (2000). Analysis of anthocyanins was performed on these crude extracts (20 μL each) by High Performance Liquid Chromatography (HPLC, Shimadzu LC-10A) equipped with PDA-detector (Shimadzu SPD-M10A*vp*) and a Waters C18 column (4.6 ϕ × 250 mm). The column was kept at 40℃ and eluted at a flow rate of 1 mL·min^{-1}; the eluate was monitored at 530 nm. Separation of anthocyanins was achieved by a linear gradient in 40 min from 20 to 85% solvent B (1.5% phosphoric acid (H_3PO_4), 20% HOAc, 25% acetonitrile in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

Isolation of anthocyanins

Fresh flowers were collected in July, from which corolla-tubes were removed and dried overnight at 37℃. The dried corolla-limbs (ca. 10 g) were extracted with 1000 mL of MAW (MeOH-HOAc-H_2O, 2:1:7, v/v/v), and the extract concentrated to 200 mL, adsorbed on Diaion HP-20 (CC), washed with 10% HOAc, and eluted with HOAc-MeOH (1:9). After this procedure the extract was concentrated again and purified by paper chromatography (PC) and thin layer chromatography (TLC) as described previously (Ando et al., 1999). Solvents used for PC and TLC were 15% HOAc and BAW (*n* - buthanol (*n* - BuOH) - HOAc - H_2O, 4:1:2, v/v/v).

Characterization of anthocyanins

Characterization of isolated pigments was carried out by TLC, HPLC (analysis conditions, see above) and Ultraviolet-Visible (UV-VIS) spectral data. TLC solvents used were BAW, BuHCl (*n*-BuOH-2N hydrochloric acid (HCl), 1:1, v/v), 1% HCl, and AHW (HOAc-HCl-H_2O, 15:3:82, v/v/v) for anthocyanins, Forestal (HOAc-HCl-H_2O, 30:3:10, v/v/v) for anthocyanidins, and BAW, EAA (ethyl acetate-HOAc-H_2O, 3:1:1,

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v/v/v), 15% HOAc and ETN (EtOH-NH_4OH-H_2O, 16:1:3, v/v/v) for sugars. Acid hydrolysis and H_2O_2 oxidation of anthocyanins were performed according to standard procedure (Harborne, 1984). Authentic commercial standard sugars were used (Wako chemicals). Sample spots on chromatograms were detected by using aniline hydrogen phthalate (AHP) spray reagent for sugars. Glucosides of delphinidin, petunidin, and malvidin were prepared from the seed coat of *Liripoe* (Ishikura and Sugahara, 1979). Delphinidin, petunidin, and malvidin were prepared by acid hydrolysis of these anthocyanins (Harborne, 1984).

Results and Discussion

The HPLC profile of anthocyanins isolated from fresh corolla-limb of 'Baccara Red' (Fig. 1A) reveal three major pigments, cyanidin 3-sophoroside (22.1% of total absorbance for all anthocyanins detected at 530 nm), cyanidin 3-glucoside (52.2%), and peonidin 3-glucoside (8.7%) were detected. These major anthocyanins have already been reported previously (Ando et al., 2000). The other three minor pigments will be referred to as pigments A (5.7%), B (1.7%), and C (1.3%).

Pigments A to C were purified from the dried corolla-limb with CC, PC, and TLC, and concentrated as described above. Acid hydrolyses of pigments A, B, and C yielded delphinidin (TLC R_f value, 0.23 (Forestal); HPLC retention time (t_R) (min), 19.4), petunidin (R_f, 0.39; t_R, 26.2), and malvidin (R_f, 0.56; t_R, 33.0) as the aglycones, respectively; glucose (TLC R_f values; 0.23 (BAW), 0.35 (EAA), 0.90 (15% HOAc), 0.67 (ETN), brown coloration with AHP) was the principal sugar component. Based on the R_f-values on TLC, t_R (min), the spectral properties by PDA on HPLC, UV-VIS spectral properties, and the results of direct comparison with the authentic anthocyanins (Table 1), pigments A, B, and C were determined as delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside, respectively. The occurrence of malvidin 3-glucoside (pigment C) and petunidin 3-glucoside (pigment B) were recorded for the first time in the genus *Petunia*.

Ando et al. (2000) who analyzed floral anthocyanins of red petunias identified delphinidin 3-glucoside (pigment A) but could not separate cyanidin 3-sophoroside by HPLC. In our study, we readily detected delphinidin 3-glucoside (pigment A) as a separate compound, possibly because of improved HPLC operation conditions.

Methylation of the B-ring (Fig. 1B) of anthocyanin to synthesize peonidin, petunidin, or malvidin is the last modification step of the biosynthesis in this plant (Cornu and Maizonnier, 1983; Gerats and Martin, 1992; Holton and Cornish, 1995; Tanaka et al., 1998; Wiering and de Vlaming, 1984). Anthocyanin 3'-methyltransferase (A3'MT) as well as 3', 5'-methyl-transferase

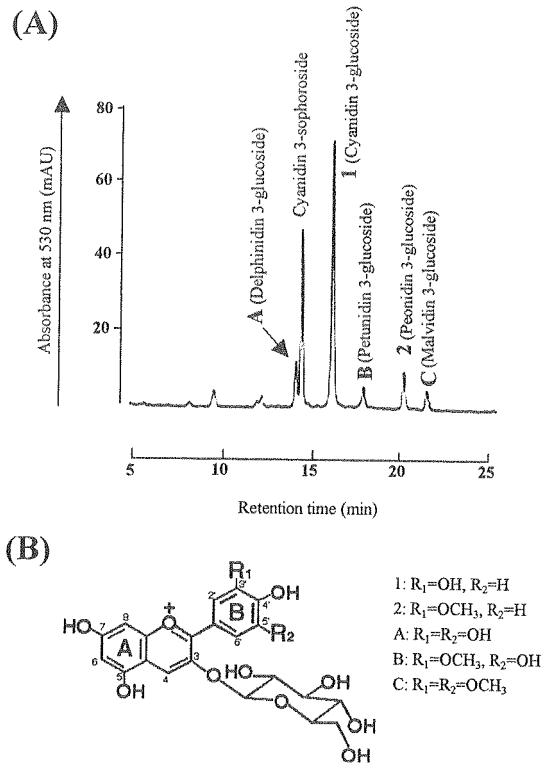

Fig. 1. HPLC profile (A) and structures (B) of anthocyanins isolated from the fresh corolla-limb of 'Baccara Red'.

Table 1. Chromatographic and spectral properties of anthocyanins isolated from petunia, 'Baccara Red'

Anthocyanin	R_f values (× 100)				Spectral data in 0.1%HCl-MeOH			HPLC		
	BAW	BuHCl	1%HCl	AHW	λ_{max} (nm)	E_{440}/E_{max} (%)	$AlCl_3$	λ_{max} (nm)	E_{440}/E_{max} (%)	t_R (min)
A	20	2	3	9	541, 280	18	+	524, 276	28	14.2
B	23	3	3	14	540, 279	20	+	526, 276	26	18.1
C	25	4	3	18	538, 280	21	-	528, 277	25	22.2
Delphinidin 3-glucoside[z]	20	2	3	9	541, 280	18	+	524, 276	28	14.2
Petunidin 3-glucoside[z]	23	3	3	14	540, 279	20	+	526, 276	26	18.1
Malvidin 3-glucoside[z]	25	4	3	18	538, 280	21	-	528, 277	25	22.2
Cyanidin 3-sophoroside[y]	31	22	36	57	528, 281	32	+	516, 280	33	14.5
Cyanidin 3-glucoside[y]	35	24	7	22	529, 282	27	+	517, 279	31	16.3
Peonidin 3-glucoside[y]	39	27	8	27	528, 281	28	-	518, 279	31	20.6

[z] Anthocyanins from the seed coat of Liriope (Ishikura and Sugahara, 1979).
[y] Standarad anthocyanins (Ando et al., 2000).

(A3'5'MT) of petunia preferably use 3-acyl-rutinoside-5-glucoside of cyanidin, delphinidin or petunidin as the substrate (Jonsson et al., 1982, 1983a, b, 1984). However, 3-acyl-rutinoside-5-glucoside of anthocyanin was not detectable in the flowers of red petunia in this study. Thus, the substrate specificity of petunia A3'MT and A3'5'MT may be incomplete; these enzymes may use non-acylated anthocyanins as the substrate, too. Alternatively, A3'MT and A3'5'MT which have different substrate specificities should be considered. If A3'MT or A3'5'MT of different substrate specificities are available, additional anthocyanins may occur in the petunia cultivars.

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赤花ペチュニア品種における微量アントシアニンの同定

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摘　要

ペチュニア'バカラレッド'の花から微量アントシアニンを単離し，クロマトグラフィーおよびスペクトル分析により，ペチュニア属では未報告のペチュニジン3-グルコシドとマルビジン3-グルコシドを同定した．これらのアントシアニンの蓄積は，ペチュニアのアントシアニン3'-および3', 5'-メチルトランスフェラーゼの不完全な基質特異性が一因と推定された．