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Anthocyanin Pigmentation Controlled by *speckled* and *c-1* Mutations of Japanese Morning Glory

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Anthocyanin pigments and their related compounds were examined in the flowers of F_1 and F_2 plants generated by crosses between two acyanic lines, 54Y having a *speckled* mutation for pale yellow flowers and 78WW*c-1* carrying the *c-1* mutation conferring white flowers, in Japanese morning glory. The mutable *speckled* allele generates speckled flowers when a dominant genetic element, *speckled-activator*, is present in its genome. The *Speckled* and *C-1* loci are tightly linked, and 78WW*c-1*, but not 54Y, bears the *specked-activator*. The flower color of F_1 progeny is red-purple, and F_2 population displays a ratio of 8 red-purple flowers: 4 white flowers: 3 speckled flowers with red-purple spots pale-yellow background: 1 pale-yellow flower. The anthocyanin components of red-purple flowers in F_1 and F_2 plants as well as red-purple spots of the speckled flower in the F_2 generation were identical. Wedding Bells Anthocyanin (WBA) was accumulated in red-purple flowers as the dominant anthocyanin along with its precursor, pelargonidin 3-sophoroside-5-glucoside, and eight of its acylated derivatives as minor anthocyanins. The major flavonoid pigment in the pale-yellow flowers of 54Y and the F_2 plants as well as the pale-yellow background of the speckled flower in the F_2 generation was identified as chalcononaringenin 2'-glucoside by chemical and spectroscopic methods. Furthermore, chlorogenic acid and small amounts of caffeic acid as well as aureusidin 4-glucoside were obtained from the pale-yellow flowers. Chlorogenic acid and caffeic acid were also detected in the white flowers of F_2 and 78WW*c-1*. These results suggest that anthocyanin biosynthesis in *speckled* and *c1* mutants stopped at the steps mediated by chalcone isomerase (CHI) and chalcone synthase (CHS), respectively, and that anthocyanin biosynthesis was completely recovered in the red-purple tissue parts of the speckled flower. Moreover, we found that anthocyanin composition varied, especially in the red-purple flowers and red-purple tissue parts in the speckled flowers of F_2 plants, implying that WBA biosynthesis depends on unknown genetic backgrounds.

Key Words: acylated pelargonidin glycoside, anthocyanin biosynthesis, chalcone isomerase, Japanese morning glory, speckled flower pigmentation.

Introduction

The Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*) has various flower color lines, and considerable attention has been paid to elucidate the chemical and genetic basis of various flower pigmentations (Hagiwara, 1954, 1956; Iida et al., 1999, 2004; Morita et al., 2005; Saito et al., 2005). So far, 44 floral anthocyanidin glycosides of pelargonidin, cyanidin, and peonidin have been isolated from their cyanic flowers, and their structures determined (Ishikura, 1981; Lu et al., 1991, 1992a, b; Saito et al., 1993, 1994b, 2005; Shibata and Yoshitama, 1969; Toki et al., 2001a, b, 2004). As compounds related to anthocyanins, quercetin 4'-glucoside, quercetin 7-glucoside, chalcononaringenin, aureusidin 4-glucoside, *R*- and *S*-naringenin 7-glucosides have also been isolated from the flowers of acyanic lines (Saito et al., 1994a).

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The recessive *speckled* mutant of the Japanese morning glory produces flowers with fine and round colored spots on a pale yellow background. It has been studied classically by Imai (1921, 1927, 1931, 1934) and Hagiwara (1956), and the *speckled* allele is thought to carry a transposon (Abe et al., 1997). Subsequently, Abe et al. (1997) carried out a cross between a *speckled* mutant of the 54Y line with a pale-yellow flower and a *c-1* mutant of the 78WW*c-1* line with a white flower, and observed that the progeny of its F_1 hybrid had all red-purple flowers. They also reported that four distinct classes with respect to the flower colors, red-purple, white, speckled with red-purple spots on a pale-yellow background, and paleyellow, were segregated in agreement with the ratio of 8 : 4 : 3 : 1 in F_2 progenies (Fig. 1). From these results, it was suggested that the appearance of variegated phenotypes in the *speckled* mutant was controlled not only by the recessive *speckled* allele but also by a dominant genetic element, termed *speckled-activator*. The *speckled-activator* was supposed to be an autonomous element acting in trans on a non-autonomous element in the *speckled* allele (Abe et al., 1997). Further analysis revealed that the *c-1* mutation is a 2 bp deletion in the gene that encodes a transcriptional activator with the R2R3-MYB domain for anthocyanin biosynthesis genes, including genes for chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) (Morita et al., 2006).

While genetic studies of the mutable *speckled* line is well advanced, as mentioned above, the chemical characterizations of anthocyanins on speckled flowers of the F_2 generation in the cross between 54Y and 78WW*c-1* lines and those of related hybrid progeny remain to be elucidated. In this study, anthocyanins and their related components were analyzed for the speckled flowers of F_2 progeny obtained by crosses between 54Y

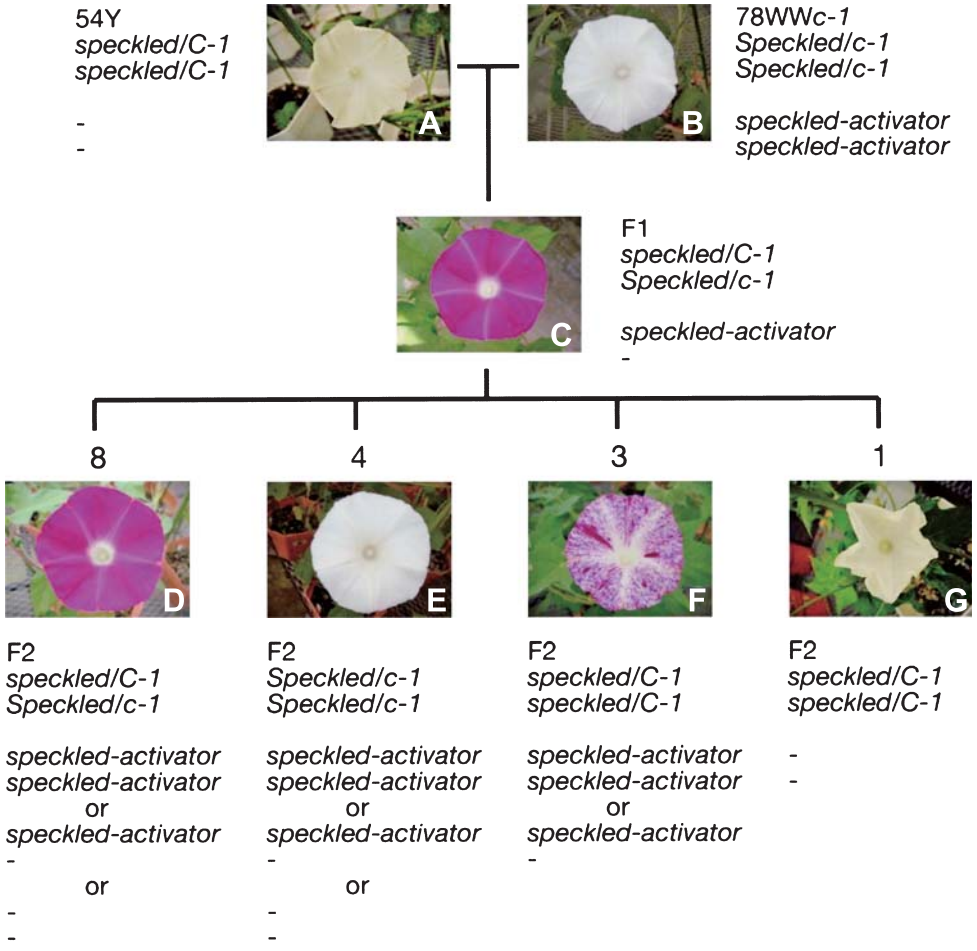

Fig. 1. Flower color phenotypes and genotypes of the lines 54Y and 78WW*c-1*, and progeny of the hybrids between these lines. A: The 54Y line has pale-yellow flowers. B: The 78WW*c-1* line has white flowers and red stems. C: The F_1 hybrid between 54Y and 78WW*c-1* lines has red-purple flowers. D, E, F, and G: Four phenotypes in F_2 progeny are exhibited. D: Red flower. E: White flower with red stem. F: Speckled flower showing fine and round spots on a pale-yellow background with variegated stems by colored sectors and spots. G: Pale-yellow flower with green stem. Numerals present the ratio of each flower phenotype in F_2 segregants. To show speckled phenotypes, the plant must carry the recessive *speckled* allele homozygously and at least one copy of the dominant *speckled-activator*. - (hyphen): absence of *speckled-activator*. The *Speckled* and *C-1* loci are tightly linked and have been mapped near the end of linkage group III (Imai, 1929, 1938; Hagiwara, 1956, 1977).

and 78WW*c-1* lines as well as red-purple, white, red-purple spots on a pale-yellow background, and pale-yellow flowers in F_2 progeny. Furthermore, the flavonoid components were reinvestigated for the flowers of 54Y and 78WW*c-1* lines.

Materials and Methods

Plant materials

The 54Y and 78WW*c-1* lines of Japanese morning glory were from our collection (Abe et al., 1997; Fukada-Tanaka et al., 1997; Morita et al., 2006). The 54Y line carries the mutable *speckled* allele homozygously without the *speckled-activator*, and exhibits a pale-yellow flower with green stems and pigmented seeds (Abe et al., 1997; Hoshino et al., 1997). The 78WW*c-1* line carries the *speckled-activator* and a recessive *c-1* allele homozygously, and exhibits a white flower with red stems and pigmented seeds (Abe et al., 1997; Fukada-Tanaka et al., 1997; Morita et al., 2006). They were grown in the fields of Minami-Kyushu University (Takanabe, Miyazaki, Japan) and Chiba Universty (Matsudo, Chiba, Japan), in the greenhouses of the National Institute for Basic Biology (Okazaki, Aichi, Japan), and in the garden of Dr. Y. Abe (Mishima, Shizuoka, Japan).

To obtain the seeds of F_1 and F_2 plants, crosses between 54Y and 78WW*c-1* lines as well as their reciprocal cross were performed in the fields and garden mentioned above. F_1 plants produced all red-purple flowers, and their stems and seeds were pigmented. In the F_2 plants generated by the self-ferilization of F_1 plants, we observed four classes of segregants displaying red-purple flowers, white flowers, red-purple speckled flowers with a pale-yellow background, and pale-yellow flowers in a ratio of 8 : 4 : 3 : 1, respectively (Abe et al., 1997) (Fig. 1). These flowers were collected from late summer to autumn from these fields, dried overnight at 45°C, and kept in a refrigerator at about 4°C, except for some fresh flowers that were directly used to extract pigments in order to examine the components and amounts of these pigments in fresh flowers.

The anthocyanin-containing tissues of the speckled flower were cut from the petal and then immersed in extract solvent, MAW (MeOH/HOAc/H_2O = 4 : 1 : 5, v/v/v). Subsequently, anthocyanins were extracted and analyzed as follows.

General chemical experimental procedure

Thin layer chromatography (TLC) was carried out on microcrystalline cellulose sheets (Merck, Germany) using mobile phases: BAW (*n*-BuOH/HOAc/H_2O, 4 : 1 : 2, v/v/v), BuHCl (*n*-BuOH/2N HCl, 1 : 1, v/v, upper layer), AHW (HOAc/HCl/H_2O, 15 : 3 : 82, v/v/v), and 1% HCl for anthocyanins; BAW, Forestal (HCl/HOAc/H_2O, 3 : 30 : 10, v/v/v), 15% HOAc, and PhOH (water-saturated phenol) for other flavonoids and caffeic acid deriva-tives; BAW, *i*-ProOH/*n*-BuOH/H_2O (7 : 1 : 2, v/v/v), and PhOH for sugars; and BAW, EtOAc/HOAc/H_2O (3 : 1 : 1, v/v/v), and EtOH/H_2O/NH_4OH (16 : 3 : 1) for organic acids (Tatsuzawa et al., 2010a, b, c).

The sample spots on TLC were detected using a UV-light lamp for flavonoids and caffeic acid derivatives, and an aniline hydrogen phthalate spray reagent for reducing sugars (Harborne, 1984). Analytical high performance liquid chromatography (HPLC) was performed with LC-10A and LC-6A pump systems (Shimadzu Co., Ltd., Japan) using a C18 (4.2 × 250 mm, Waters, USA) column at 40°C with a flow rate of 0.8 ml/min, and monitored at 530 nm for anthocyanins (LC-10A system), at 400 nm for chalcones and aurones (LC-6A system), at 380 nm for flavonols (LC-6A system), and at 320 nm for flavonols and caffeic acid derivatives (LC-6A system). The eluant was applied as a linear gradient system for 40 min for 25% to 80% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

UV-VIS spectra were recorded on an MPS-2450 (Shimadzu) in 0.1% HCl-MeOH for anthocyanins and in MeOH for other compounds. FAB mass spectra were obtained in positive ion mode using a magic bullet (5 : 1 mixture of dithiothreitol and dithioerythritol) as a matrix by JEOL JMS SX-102A (JEOL Ltd., Japan). NMR spectra were measured for ^1H spectra at 500 MHz and ^{13}C spectra at 125.78 MHz in DMSO-d_6 by JEOL JMN GX-500 (JEOL Ltd.). Chemical shifts were reported relative to TMS internal standard (δ), and coupling constants are in Hz.

Anthocyanin analysis

Anthocyanin pigments (1–10) were extracted from the flowers (ca. 100 g) of F_1 and F_2 plants, with 5% HOAc or MAW solvent (500 mL), at room temperature for 10 h. The reddish-purple concentrated extract was subjected to an ion exchange column chromatography (CC) (Diaion HP-20, Mitsubishi Chemical, Japan), and the column was washed with H_2O. The anthocyanin pigments (1–10) were eluted with MeOH/HOAc/H_2O (75 : 5 : 20, v/v/v). After concentration, the eluated were fractionated with Sephadex LH-20 CC (Amersham Biosciences, Sweden) using MeOH/HOAc/H_2O (6 : 1 : 12, v/v/v). The fractions were further isolated and purified with paper chromatography (BAW and 15% HOAc), and preparation HPLC, which was performed on a C18 (19 × 150 mm, Waters) column at 40°C with HOAc solvent and a flow rate of 4 mL·min^{-1}, and monitored at 530 nm. Ten anthocyanin pigments 1–10 (ca. 1–7 mg, Table 1) were obtained and used to analyze their structures. At the same time, the red-purple tissue parts of speckled flower were cut from the pale-yellow tissue parts and immersed in 5% HOAc and/or MAW solvent. Anthocyanin pigments 1–10 were extracted, isolated and purified by the same processes as for the red-purple flowers.

Other flavonoid analysis

The pale-yellow flowers (ca. 200 g) of F_2 plants were immersed in hot 80% MeOH solvent (1L) for 60 min or 50% MeOH solvent at room temperature for 3 h. The extract was concentrated to 1/10 volume and passed through a Sephadex LH-20 CC with MAW solvent. By this process, chalcononaringenin 2'-glucoside, aureusidin 4-glucoside, chlorogenic acid, and caffeic acid were fractionated and isolated with MAW solvent. These fractions of compounds were evaporated in vacuo to small volumes.

These substances were further purified by TLC with BAW and 15% HOAc. After the above processes, chalcononaringenin 2'-glucoside (10 mg) and chlorogenic acid (7 mg) were obtained, as well as small amounts of auresidin 4-glucoside and caffeic acid. These compounds were used for structure determination (Table 2). The structure of chalcononaringenin 2'-glucoside was also confirmed by analysis of ^{1}H and ^{13}C NMR spectra (Table 3).

By a similar process, chalcononaringenin 2'-glucoside, chlorogenic acid, auresidin 4-glucoside, and caffeic acid were isolated from the pale-yellow tissue parts of speckled flowers and the pale-yellow corollas of the 54Y line, and purified. Chlorogenic acid and caffeic acid were isolated from the white flowers of F_2 with 50% MeOH solvent, and purified by PC and TLC with BAW and 15% HOAc.

Distribution survey of anthocyanins and related compounds in the flowers of F_1 and F_2 plants

Fresh corolla limbs or tissues (ca. 0.02–0.10 g each) of the flowers were immersed in 50% MeOH (10 mL) containing 1.5% H_3PO_4 or MAW for 60 min, and their anthocyanin pigments and related compounds were thoroughly extracted. These anthocyanin pigments and

Table 1. Chromatographic and spectroscopic data of anthocyanins isolated from the red-purple flowers of F_1 and F_2 hybrid progenies crossed between 54Y and 78WW*c-1*.

Anthocyanin[z]	Rf value (×100)				HPLC	Spectral data [λ_{max} (nm)] in 0.1% HCl-MeOH	FAB-MS
	BAW	BuHCl	1%HCl	AHW	Rt (min)		$[M]^+$
1	35	16	61	74	11.2	507, —, 268	757
2	31	14	43	66	17.6	506, 322, 284	1081
3	17	8	15	32	20.3	505, 330, 285	919
4	51	35	26	54	26.1	504, 319, 284	903
5	43	23	23	49	30.6	510, 326, 288	1243
6	48	30	41	63	32.5	509, 331, 288	919
7	58	12	25	41	33.0	510, 329, 288	1081
8	24	9	14	36	33.5	515, 318, 288	1729
9	43	3	16	33	35.2	512, 319, 287	1567
10	49	3	7	28	37.6	511, 325, 289	1405

For key to abbreviations, see "Materials and Methods": BAW = *n*-BuOH-HOAc-H_2O (4 : 1 : 2), BuHCl = *n*-BuOH-2M HCl (1 : 1), AHW = HOAc-HCl-H_2O (15 : 3 : 82).

[z] Anthocyanin numbers are the same as those of Figure 2 and Table 4.

1: pelargonidin 3-sophoroside-5-glucoside.
2: pelargonidin 3-[2-(glucosylcaffeoylglucosyl)-glucoside]-5-glucoside.
3: pelargonidin 3-[2-(caffeoylglucosyl)-glucoside]-5-glucoside.
4: pelargonidin 3-[2-(*p*-coumaroylglucosyl)-glucoside]-5-glucoside.
5: pelargonidin 3-[2-(glucosylcaffeoylglucosyl)-6-(caffeoyl)-glucoside]-5-glucoside.
6: pelargonidin 3-[2-(glucosyl)-6-(caffeoyl)-glucoside]-5-glucoside.
7: pelargonidin 3-[2-(caffeoylglucosyl)-6-(caffeoyl)-glucoside]-5-glucoside.
8: pelargonidin 3-[2-(glucosylcaffeoylglucosyl)-6-(glucosylcaffeoylglucosylcaffeoyl)-glucoside]-5-glucoside (Wedding Bells Anthocyanin (WBA)).
9: pelargonidin 3-[2-(glucosylcaffeoylglucosyl)-6-(caffeoylglucosylcaffeoyl)-glucoside]-5-glucoside.
10: pelargonidin 3-[2-(caffeoylglucosyl)-6-(caffeoylglucosylcaffeoyl)-glucoside]-5-glucoside.

Table 2. Rf values and spectral data of flavonoids and related components in the acyanic flowers of 54Y line, 78WW*c-1* line and their F_2 hybrid progenies.[z]

Compound[y]	Rf value (×100)			HPLC	UV-VIS spectral data	Color in	
	BAW	15% HOAc	PhOH	Rt (min)	λmax (nm) in MeOH	UV	UV + NH_3
chalcononaringenin 2'-glucoside	63	17	56	21.3	365	dark brown	yellow
aureusidin 4-glucoside	47	4	41	15.8	(405), 271, 255	bright yellow	bright orange-red
chlorogenic acid	69	66	62	6.0	328, 288, (242)	blue	green
caffeic acid	84	38, 68	53	7.3	325, 234	blue	light blue

[z] For key to solvent mixture, see "Materials and Methods". ( ): inflection on shoulder.

[y] chalcononaringenin 2'-glucoside = pigment 12, Aureusidin 4-glucoside = pigment 11.

Table 3. NMR spectroscopic data of chalcononaringenin 2'-glucoside (pigment 12) isolated from pale-yellow flowers.

	^{13}C δ (ppm)	^{1}H δ (ppm)	
chalcononaringenin			
1	128.1		
2	126.1	7.63	d (8.3)
3	124.1	6.82	d (8.3)
4	157.5		
5	124.1	6.82	d (8.3)
6	126.1	7.63	d (8.3)
1'	105.0		
2'	166.1		
3'	94.9	6.13	d (1.9)
4'	172.5		
5'	100.2	5.90	d (1.9)
6'	160.2		
α	115.0	7.99	d (15.6)
β	142.4	7.59	d (15.6)
CO	191.5		
glucose			
1	97.0	5.06	d (7.7)
2	73.6	3.29	dd (7.7, 8.6)
3	77.2	3.23	t (9.0)
4	69.3	3.36	t (8.9)
5	76.7	3.32	m
6a	60.6	3.52	dd (5.2, 11.3)
6b		3.71	d (11.3)

^{13}C: 125.78 MHz in DMSO-d_6 and ^{1}H: 500 MHz in DMSO-d_6 with an internal standard of TMS. Coupling constants (J in Hz) in parentheses.

related compounds in the extracts were analyzed by TLC and HPLC methods (Saito et al., 1994b). From the data of HPLC analysis, the relative frequency of a pigment's occurrence in the extracts was observed by HPLC: Waters C18 (4.6 × 250 mm) column at 40°C with a flow rate of 1 mL·min^{-1} monitored at 530 nm for anthocyanins, at 400 nm for chalcone and aurone, at 320 nm for caffeic acid derivatives, and 380 nm for flavonols.

Results and Discussion

Phenotypes and genotypes of the speckled and c-1 mutants and progeny in the hybrids

The flowers of F_1 hybrids, which were obtained from reciprocal crosses between 54Y and 78WW*c-1* carrying *speckled* and *c-1* mutations, respectively, were all red-purple (Fig. 1). The F_2 hybrid plants by self-pollination with F_1 hybrid plants were segregated into four groups described previously by Abe et al. (1997), that is, in the ratio of 8 (red-purple flower); 4 (white flower): 3 (speckled flower; red-purple fine and round spots on a pale-yellow back-ground): 1 (pale-yellow flower), respectively (Fig. 1). The flowers of these F_2 plants were used as analytical materials of floral pigments as follows.

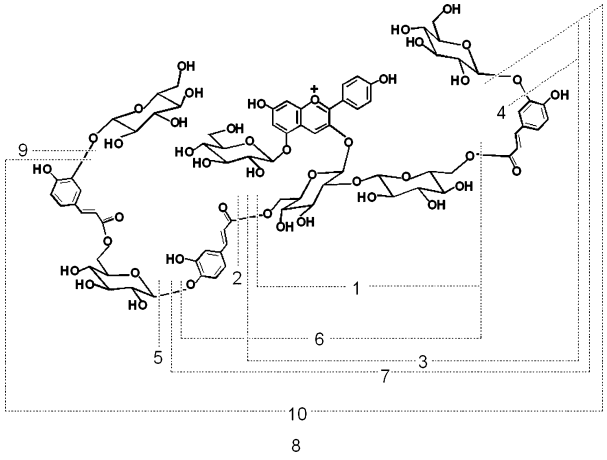

Fig. 2. Wedding Bells Anthocyanin (pigment 8) and other anthocyanins (pigments 1–7, 9, and 10) in the red-purple flowers and the speckled flower of Japanese morning glory.

Anthocyanin components of red-purple flowers in F_1 and F_2 plants

Ten anthocyanin peaks (pigments 1–10) were observed in 5% acetic acid extracts from the red-purple flowers of F_1 and F_2 plants by HPLC analysis (Figs. 2 and 3A, Table 1). The relative percentages of their occurrence in F_1 flowers were 1.8–3.3% (pigment 1), 1.5–5.0% (pigment 2), 1.4–3.4% (pigment 3), 0–1.9% (pigment 4), 1.5–8.5% (pigment 5), 0–2.3% (pigment 6), 0–1.7% (pigment 7), 66.8–80.7% (pigment 8), 2.0–8.9% (pigment 9), and 1.6–2.3% (pigment 10), respectively, as shown in Table 4. These results of ten anthocyanin peak distributions were almost identical to those of common red-purple flower cultivars such as 'Scarlet O'Hara', 'Violet', and *dingy* mutant (Lu et al., 1992a; Toki et al., 2001a, b). The anthocyanin constitutions of F_2 plants were almost identical to those of red-purple F_1 plants, but their relative distribution values rather varied in comparison with those of F_1 plants (Table 4). In particular, the contents (4.0–20.4% and 1.4–27.3%) of pigments 7 and 5 in F_2 plants were greater than those (0–1.7% and 1.5–8.5%) of the red-purple flowers of F_1 plants. The variability of the anthocyanin composition will be discussed below.

These ten anthocyanins (pigments 1–10) were isolated from the red-purple flowers of F_1 and F_2 plants with 5% HOAc as the extract solvent, and purified using Diaion HP-20 CC, prep. HPLC, and TLC according to the procedure described previously (Toki et al., 2001a). The chromatographic and spectroscopic properties of these pigments are summarized in Table 1. Upon acid hydrolysis, all ten pigments 1–10 gave pelargonidin and glucose and, except for pigments 1 and 4, eight pigments, 2, 3, and 5–10, gave caffeic acid as their acid component. Pigment 4 gave *p*-coumaric acid as its acid. Upon alkaline hydrolysis, the pigments (2–10) gave pelargonidin 3-sophoroside-5-glucoside, which is identical to pigment 1. By chromatographic and spectroscopic

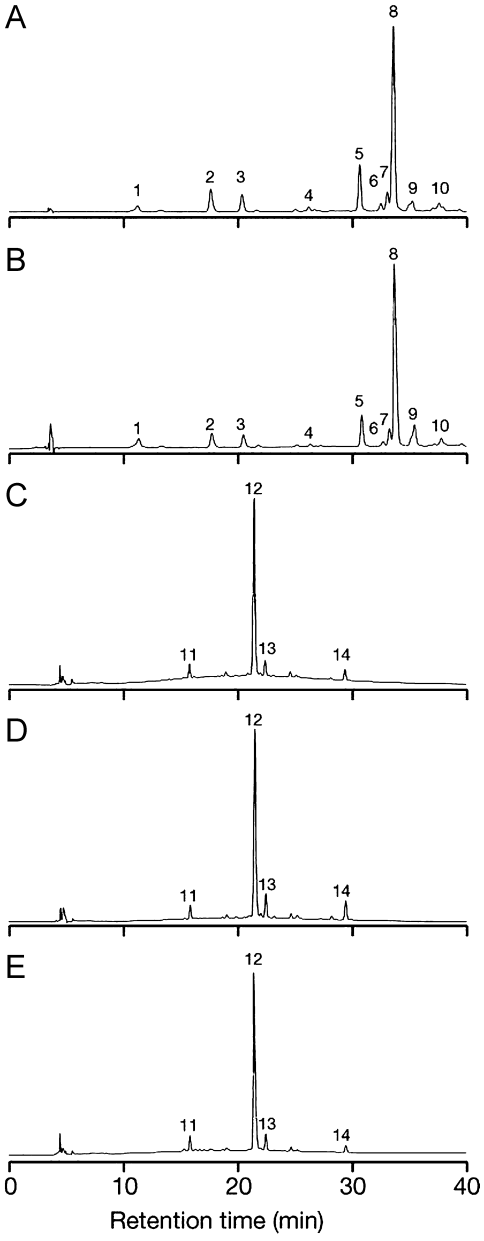

Fig. 3. Comparative HPLC profiles of anthocyanin extracts (530 nm) and chalcone extracts (400 nm) from the flowers of Japanese morning glory hybrids.
A: Anthocyanin extract of F_2 red-purple flower with MAW (MeOH/Acetic acid/Water). Anthocyanin pigment numbers are the same as those in Table 1.
B: Anthocyanin extract of F_2 red-purple tissue parts of the speckled flower in F_2 generation with MAW. Anthocyanin pigment numbers are the same as those in Figure 3A.
C: Chalcone extract from the F_2 pale-yellow flower with aqueous MeOH. Pigment 11: aureusidin 4-glucoside. Pigment 12 (12): chalcononaringenin 2'-glucoside. Pigment 13 (13): unknown. Pigment 14 (14): unknown.
D: Chalcone extract from the pale-yellow flower of 54Y line with aqueous MeOH. Yellow pigment numbers are the same as those in Figure 3C.
E: Chalcone extract from the pale-yellow tissue parts of the speckled flower in F_2 generation with aqueous MeOH. Yellow pigment numbers are the same as those in Figure 3C.

methods employing authentic anthocyanin samples, obtained from common red-purple cultivars (Lu et al., 1992a; Saito et al., 1996; Toki et al., 2001a), to be compared, these pigments 1–10 were identified to be pelargonidin 3-sophoroside-5-glucoside as pigment 1, pelargonidin 3-[2-(6-(3-(glucosyl)-caffeoyl)-glucosyl)-glucoside]-5-glucoside (Pharbitis Red Anthocyanin 1, PRA 1) as pigment 2, pelargonidin 3-[2-(6-(caffeoyl)-glucosyl)-glucoside]-5-glucoside (PRA 4) as pigment 3, pelargonidin 3-[2-(6-(*p*-coumaroyl)-glucosyl)-glucoside]-5-glucoside (PRA 7) as pigment 4, pelargonidin 3-[2-(6-(3-(glucosyl)-caffeoyl)-glucosyl)-6-(caffeoyl)-glucoside]-5-glucoside (PRA 3) as pigment 5, pelargonidin 3-[2-(glucosyl)-6-(caffeoyl)-glucoside]-5-glucoside (PRA 2) as pigment 6, pelargonidin 3-[2-(6-(caffeoyl)-glucosyl)-6-(caffeoyl)-glucoside]-5-glucoside (Ipomoea Red Anthocyanin 4, IRA 4) as pigment 7, pelargonidin 3-[2-(6-(3-(glucosyl)-caffeoyl)-glucosyl)-6-(4-(6-(3-(glucosyl)-caffeoyl)-glucosyl)-caffeoyl)-glucoside]-5-glucoside (WBA) as pigment 8, pelargonidin 3-[2-(6-(3-(glucosyl)-caffeoyl)-glucosyl)-6-(4-(6-(caffeoyl)-glucosyl)-caffeoyl)-glucoside]-5-glucoside (IRA 1) as pigment 9, and pelargonidin 3-[2-(6-(caffeoyl)-glucosyl)-6-(4-(6-(caffeoyl)-glucosyl)-caffeoyl)-glucoside]-5-glucoside (IRA 2) as pigment 10, respectively (Fig. 2 and Table 1). These structures were confirmed by measuring their FAB mass spectra (Table 1).

Flavonoids and their related components in pale-yellow flowers in F_2 generation and 54Y line

As shown in the HPLC profile (Fig. 3C) of the aqueous MeOH extract from pale-yellow flowers (Fig. 1) in F_2 plants, pigment 12 was observed as the dominant yellow pigment along with small amounts of three other pigments, 11, 13, and 14. Pigments 12 and 14 were isolated from the aqueous MeOH extract of pale-yellow flowers of F_2, and purified by Sephadex CC and TLC. Chlorogenic acid and small amounts of caffeic acid were also obtained from the extract solution. The chromatographic and spectroscopic properties of these compounds are summarized in Table 2. From these results, we were able to identify and confirm chlorogenic acid and caffeic acid by comparing with authentic samples available commercially.

Pigment 12 was presumed to be chalcononaringenin 2'-glucoside from the results of TLC and spectroscopic properties. Its exact structure was determined by measuring its FAB mass spectrum 434.121 ($C_{21}H_{22}O_{10}$) and ^1H and ^{13}C NMR spectra, as shown in Table 3 and Figure 4.

Pigment 11 was presumed to be aureusidin 4-glucoside by its chromatographic and spectroscopic properties (Table 2), but its structure was not elucidated further due to the small amounts obtained. From these results, therefore, chalcononaringenin 2'-glucoside (pigment 11) must be responsible for the yellow coloration of this pale-yellow flower in F_2 plants.

Table 4. Typical anthocyanin distributions in red-purple flowers of F_1 and F_2 and red-purple tissue parts of speckled flower of F_2 progenies.

Hybrid progeny: cross types and reference numbers	Anthocyanin (as %)[z]									
	1	2	3	4	5	6	7	8	9	10
F_1 red-purple flower										
(1) 78WW*c-1* × 54Y										
1	1.9	3.4	1.7	1.2	8.5	0.7	1.7	69.5	2.5	1.6
2	2.5	5.0	3.4	1.6	5.7	2.2	—	66.8	8.2	2.2
3	2.4	3.0	2.3	1.0	4.5	2.2	—	73.9	7.0	1.9
4	2.0	2.0	2.2	—	1.5	—	—	80.7	7.3	1.9
(2) 54Y × 78WW*c-1*										
1	2.9	1.5	1.4	0.8	4.6	0.7	0.9	73.3	3.7	1.6
2	1.8	2.4	1.7	1.1	3.6	1.7	—	80.4	2.0	2.3
3	3.3	4.6	2.8	1.9	3.1	1.6	—	75.3	2.4	2.3
4	2.5	4.2	1.8	1.1	5.3	2.3	—	69.2	8.9	2.3
F_2 red-purple flower										
1	2.0	2.0	2.1	1.1	12.7	3.1	6.5	53.9	4.9	2.8
2	2.4	6.5	4.7	1.4	12.1	2.2	4.7	49.4	2.9	2.7
3	1.1	4.5	2.2	1.4	21.1	2.9	8.8	38.6	3.7	3.5
4	1.9	5.3	4.9	1.1	25.2	4.5	20.4	23.3	2.5	5.4
5	0.2	0.4	0.6	0.4	1.4	1.2	4.0	72.4	5.4	4.5
6	4.2	6.8	4.7	1.8	27.3	4.4	9.8	23.7	3.1	4.3
7	2.9	6.6	3.7	1.7	20.4	3.0	6.7	36.6	3.9	3.7
8	0.9	1.7	2.6	0.8	3.2	1.9	4.1	62.7	4.3	4.7
Red-purple tissue parts of the speckled flowers										
1	4.2	4.8	4.0	0.6	8.8	1.4	4.8	55.1	9.0	2.9
2	1.0	0.5	2.7	—	1.6	0.6	1.7	78.9	2.0	0.5
3	1.0	2.9	2.7	—	4.3	—	0.5	82.0	4.5	0.5
4	1.0	1.3	1.6	—	1.5	—	0.5	79.8	4.7	0.5

[z] Numbers of anthocyanins are the same as numbers of anthocyanin pigments 1–10 (see in Table 1). "—" indicate 0%.

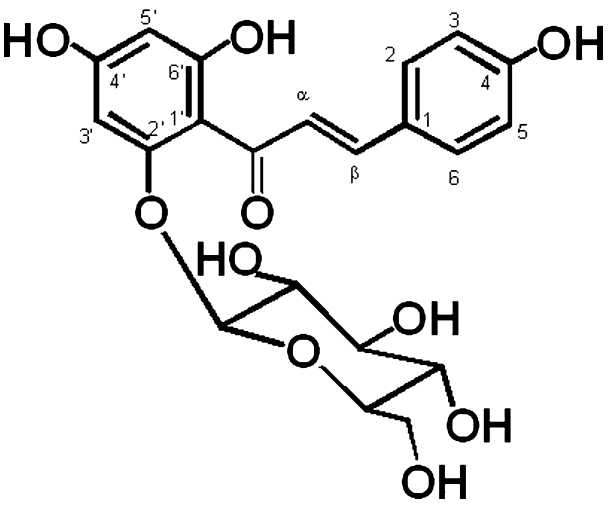

Fig. 4. Charcononaringenin 2'-glucoside (pigment 2).

As shown in Figure 3C and D, the HPLC profile of the aqueous MeOH extract from the pale-yellow flowers of the 54Y line was identical to that of the pale-yellow flowers of F_2 plants. Thus, the pigment components in the pale-yellow flowers of 54Y line were reinvestigated by the same procedures to characterize the pale-yellow flowers of F_2 plants. Two yellow pigments (pigments 11 and 12) were isolated from the extract of the pale-yellow flowers of the 54Y line, and both pigments were identified to be the same pigments isolated from the pale-yellow flowers of F_2 plants analyzed by TLC and HPLC. Consequently, the present results that chalconon-aringenin 2'-glucoside is the dominant pigment in the pale-yellow flowers of the 54Y line differ from previous results that chalcononaringenin and naringenin 7-glucoside were the major flavonoids in the flowers of the 54Y line (Saito et al., 1994b). Presumably, chal-cononaringenin without a sugar molecule and naringenin 7-glucoside detected previously are likely to be artifacts produced enzymatically during their previous extraction processes that lasted many hours.

Flavonoids and related components in the white flowers of F_2 generation and 78WWc-1 line

Two compounds were isolated from the 50% MeOH extract of white flowers of F_2 and 78WW*c-1*, and were identified to be chlorogenic acid and caffeic acid from the results of chromatographic and spectroscopic properties (data not shown; Table 2). Both compounds were confirmed by TLC and HPLC analysis in comparison with authentic chlorogenic acid and caffeic acid. Further, by measuring the mass spectrum, the FAB mass spectrum of chlorogenic acid was determined to

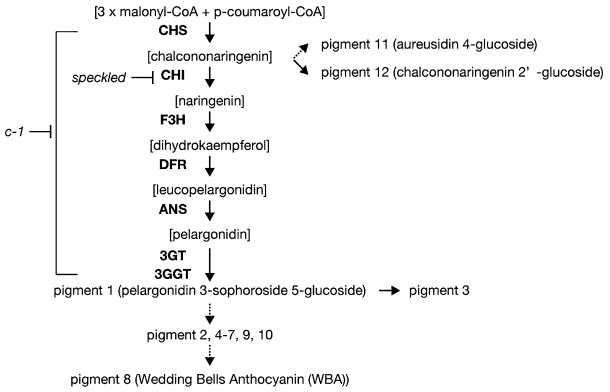

Fig. 5. Scheme illustrating the possible anthocyanin biosynthesis pathways from hydroxycinnamic acid to Wedding Bells Anthocyanin (WBA) in the red-purple flower of Japanese morning glory. Flavonoid compounds in square brackets were not detected in this study. Enzymes are in bold. CHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavanone 3-hydroxylase, DFR: dihydroflavonol 4-reductase, F3'H: flavonoid 3-hydroxylase, ANS: anthocyanidin synthase, 3GT: UDP-glucose:flavonoid 3-*O*-glucosyltransferase, 3GGT: UDP-glucose:anthocyanin 3-*O*-glucose-2"-*O*-glucosyltransferase. The pathway in the *speckled* mutant of 54Y and *c1* mutant of 78WW*c-1* is virtually stopped at the steps mediated by chalcone isomerase (CHI) and chalcone synthase (CHS), respectively.

be 355.1 m/z [M + 1], which is in good agreement with the chlorogenic acid molecular weight: $C_{16}H_{18}O_9$, 354.095. No obvious accumulation of any flavonoids could be detected in the MeOH extract of the white flowers (data not shown).

It appeared that the enzymatic reaction of CHS was almost completely inhibited or blocked in the white flowers of both F_2 and 78WW*c-1*, and hydroxycinnamic acid derivatives such as chlorogenic acid were found in these flowers as their main phenolic compounds (Fig. 5). Consistent with this notion, it was previously reported that the two *CHS* genes, *CHS-D* and *CHS-E*, are expressed in the flowers of Japanese morning glory, and that the expression of the genes was markedly reduced in the flower petals of the 78WW*c-1* line (Fukada-Tanaka et al., 1997; Morita et al., 2006). The *C-1* gene encodes a transcriptional activator containing the R2R3-MYB domain and activates the transcription of anthocyanin biosynthesis genes, including all genes for the enzymes shown in Figure 5 (Morita et al., 2006).

Flavonoids and related components in the red-purple spots of the speckled flowers of F_2

Ten anthocyanins (pigments 1–10), extracted from the red-purple spots of the speckled flower (Figs. 1 and 3B), were identified with those of red-purple flowers of F_1 and F_2 plants by chromatographic and spectroscophic analyses (Tables 1 and 4). Analysis revealed that the anthocyanin constituents of the red-purple spots were almost identical to those of the red-purple flowers of F_1 and F_2 plants. Like red-purple flowers of F_2 plants, the relative amounts of these anthocyanin percentages were rather irregular in comparison with those of red-purple flowers of F_1 plants (Table 4). In particular, the percentages of WBA (pigment 8) varied; 55.1–82.0% in the red-purple tissue parts of speckled flowers, 23.3–72.4% in the red-purple flowers of F_2, and 66.8–80.7% in the F_1 flowers. The irregularities of anthocyanin constituents might be due to differences in the unknown genetic backgrounds of the parental lines, 54Y and 78WW*c-1*. Such genetic backgrounds of F_1 plants should be more uniform than those of segregated F_2 plants and may control the acylation and glycosylation of pelargonidin 3-sophoroside-5-glucoside in the latter steps of WBA biosynthesis (Fig. 5), and the irregularities of anthocyanin constituents in F_2 flowers may be attributable to variable acylation and glycosylation activities. Because anthocyanin biosynthesis in the red-purple spots of speckled flowers operated like those of red-purple flowers of F_2 plants, the *speckled* allele in the spots must completely revert to wild-type alleles.

Flavonoids and related components on the pale-yellow background of the speckled flowers of F_2

From the pale-yellow tissue parts of the speckled flowers, chalcononaringenin 2'-glucoside, auresidin 4-glucoside, chlorogenic acid, and caffeic acid were found in the aqueous MeOH extract as its major phenolic components by TLC and HPLC analysis (Table 1 and Fig. 3E). These constituents of the phenolic components of pale-yellow tissue parts were identical to those of the pale-yellow flowers of F_2 plants and the 54Y line (Fig. 3C, E).

F_2 plants with pale-yellow flowers and the speckled flower as well as the 54Y line carry the *speckled* mutation. Among these plants, only F_2 plants with speckled flowers are thought to carry the *speckled-activator* (Abe et al., 1997) (Fig. 1) and exhibit two different pigmentation patterns in the same flower, red-purple spots and pale-yellow background. Namely, anthocyanin biosynthesis only occurred in the red-purple spots, and not in the pale-yellow F_2 flowers and pale-yellow tissue parts. From the results of the pale-yellow flowers of the F_2 and 54Y plants and the pale-yellow tissue parts of the speckled flowers, a precursor of anthocyanin biosynthesis, namely chalcononaringenin, appeared not to be converted enzymatically into a flavanone, naringenin, in the pale-yellow flower tissues, but remained in the form of chalcononaringenin 2'-glucoside as the dominant flavonoid. These observations suggested that the *speckled* mutation results in reduced of the CHI activity, and somatic reversions of the *speckled* allele in the red-purple spots of the speckled flowers cause the reactivation of CHI activity (Fig. 5). Indeed, the *speckled* allele is the *CHI* gene having an inserted transposon, and the colored spots in speckled flowers are generated by somatic reversion of the *speckled* mutation (Hoshino et al., 2001; A. Hoshino and S. Iida, unpublished).

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