Identification and Distribution of Simple and Acylated Betacyanins in the Amaranthaceae

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Red-colored plants in the family Amaranthaceae are recognized as a rich source of diverse and unique betacyanins. The distribution of betacyanins in 37 species of 8 genera in the Amaranthaceae was investigated. A total of 16 kinds of betacyanins were isolated and characterized by HPLC, spectral analyses, and MS. They consisted of 6 simple (nonacylated) betacyanins and 10 acylated betacyanins, including 8 amaranthine-type pigments, 6 gomphrenin-type pigments, and 2 betanin-type pigments. Acylated betacyanins were identified as betanidin 5-*O*-*β*-glucuronosylglucoside or betanidin 6-*O*-*β*-glucoside acylated with ferulic, *p*-coumaric, or 3-hydroxy-3-methylglutaric acids. Total betacyanin content in the 37 species ranged from 0.08 to 1.36 mg/g of fresh weight. Simple betacyanins (such as amaranthine, which averaged 91.5% of total peak area) were widespread among all species of 8 genera. Acylated betacyanins were distributed among 11 species of 6 genera, with the highest proportion occurring in *Iresine herbstii* (79.6%) and *Gomphrena globosa* (68.4%). Some cultivated species contained many more acylated betacyanins than wild species, representing a potential new source of these pigments as natural colorants.

Keywords: *Amaranthaceae; acylated betacyanins; amaranthine; betanin; colorants; HPLC*

INTRODUCTION

Betacyanins, a class of water-soluble red-violet pigments, occur only in 10 families of the order Caryophyllales (old name for Centrospermae), including the family Amaranthaceae (*1*, *2*). The presence of betacyanins in plants is mutually exclusive of the occurrence of anthocyanins, which are more widely distributed in the plant kingdom. All betacyanins are glycosylated and derive mainly from their basic structural units, that is, the aglycons betanidin and isobetanidin (the C-15 epimer). The hydroxyl groups of the latter enable the formation of glycosides that occur mostly as the 5-*O*-glucosides, for example, in betanin-type betacyanins that are a major or minor pigment component in many betacyanin-producing plants. Further glycosylation at the 5-*O*-glucosides is often found, for example, the glucuronosylglucosides in amaranthin-type betacyanins that are one of the most common pigments in the Amaranthaceae (*3*). Because the word "amaranthin" was also used for the name of a lectin or a globulin protein from *Amaranthus* (*4*, *5*), the pigment was designated "amaranthine" rather than "amaranthin" to avoid confusion (*6*, *7*).

The betacyanin pigments (well-known betalains) extracted from red beets (*Beta vulgaris*) are extensively used in the food industry (*8*). Piattelli and Minale (*1*) investigated the betacyanin distribution in seven species of the genus *Amaranthus* and nine species of five other genera in the Amaranthaceae. In previous papers (*7*, *9*, *10*) we studied the pigments from 21 genotypes of 7 species in the genus *Amaranthus*, identifying them as homogeneous betacyanins (amaranthine and isoamaranthine). *Amaranthus* betacyanins have high potential for use as colorants in some food products. Red *Amaranthus* plants have attracted considerable interest as a potential alternative source of betacyanin pigments similar to those from red beet, because some *Amaranthus* genotypes produce higher biomass and contain more betacyanins than red beet. Furthermore, *Amaranthus* plants can grow in a wider range of environments than red beet.

Acylation of anthocyanins in plants occurs frequently (*2*). Acylated anthocyanin molecules may have improved stability of anthocyanins in some plants (e.g., *Ipomoea tricolor*, *Tradescantia pallida*, and *Raphanus sativus*) (*11*−*13*). However, little research on acylated betacyanins has been reported. In earlier studies, acylated betacyanins were found in some plants in the Amaranthaceae, such as celosianins from *Celosia cristata* L. (*14*) and gomphrenins from *Gomphrena globosa* L. (*15*, *16*). Schliemann and Strack (*17*) reported that acylated betacyanins (celosianin II and lampranthin II) showed reduced racemization and enhanced color stability. The acylated betacyanins, from the important genus *Amaranthus* and some other genera in the Amaranthaceae, have not yet been reported. Since 1996 we have been conducting studies on the development and application of *Amaranthus* pigments (e.g., refs *7*, *9*, and *10*). Recently we have collected additional betacyanin-producing genotypes in the Amaranthaceae. Systematic studies of both qualitative and quantitative distribution of betacyanins in this family have been now carried out. The objective of this study was to identify and quantify the simple and acylated betacyanins present in 37 species of 8 genera in the family Amaranthaceae.

MATERIALS AND METHODS

Materials. A total of 40 betacyanin-containing genotypes were tested from 37 species of 8 genera in the Amaranthaceae

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10.1021/jf000963h CCC: $20.00
Published on Web 03/06/2001

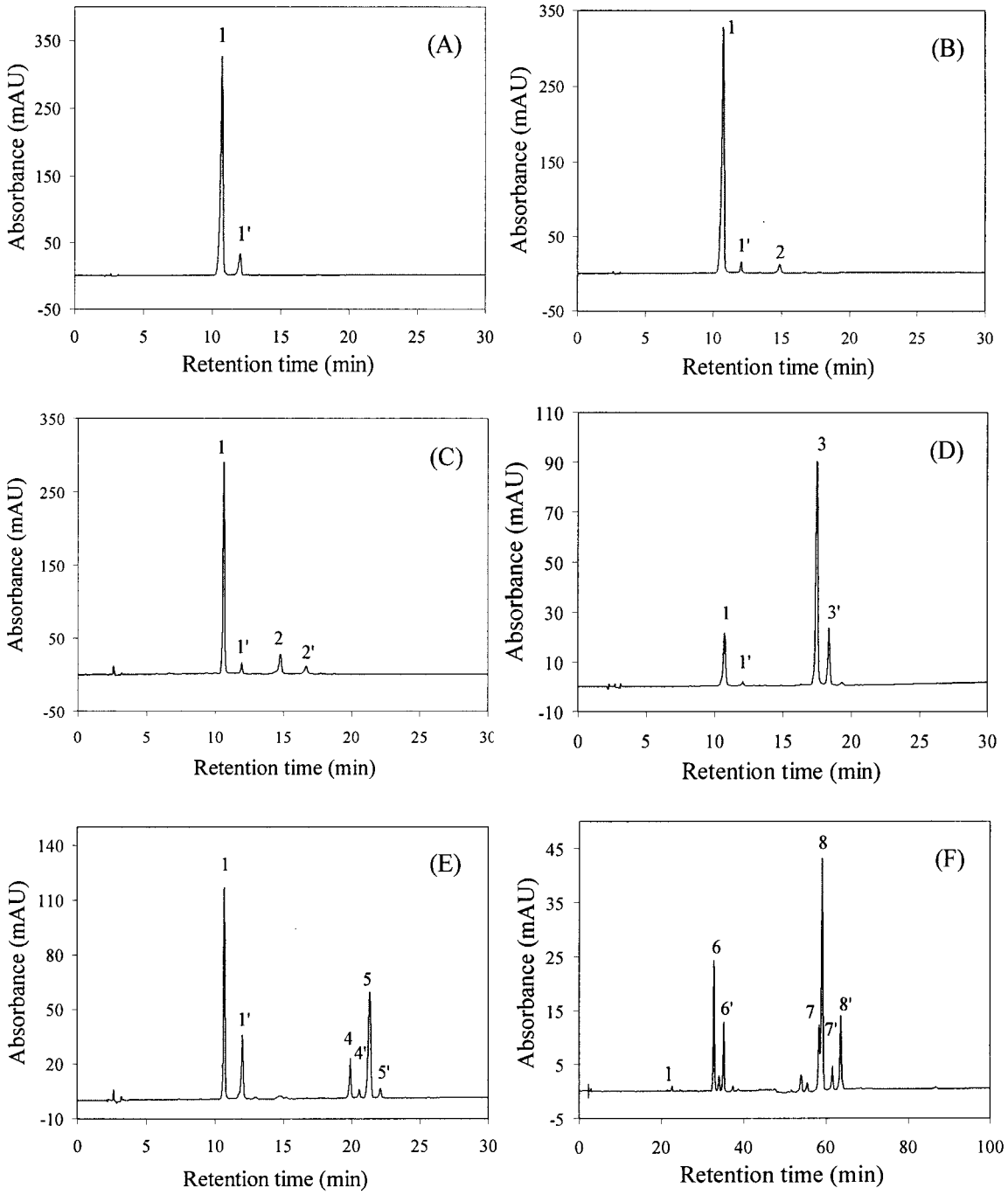

Figure 1. Typical HPLC profiles of methanolic extracts from different species in the Amaranthaceae: (A) seedlings of *A. tricolor* L.; (B) leaves of *A. cruentus* L.; (C) seedlings of *A. powellii* L.; (D) leaves of *I. herbstii* Hook. F. ex Lindl.; (E) inflorescences of *C. cristata* L. var. (violet); (F) inflorescences of *G. globosa* L. Elution was monitored at 540 nm. Linear gradient elution (30 min) was used for (A−E); 100 min was used for (F). Numbers on peaks refer to betacyanins listed in Table 1 and Figure 2.

(listed in Table 2) from 16 countries and regions, provided by the U.S. Department of Agriculture−Agricultural Research Service (USDA-ARS), Iowa State University, the Chinese Academy of Agricultural Sciences (CAAS), Beijing, and the Hubei Academy of Agricultural Sciences (HAAS), Wuhan, China. Most of the genotypes were grown in a greenhouse in Wuhan, China, in 2000, and a few genotypes were planted in a field in Wuhan in 1999.

Extraction and Purification. Samples of fresh plant materials (seedlings, leaves, inflorescences, and stems) were harvested, frozen, and kept at −18 °C until extraction. In our previous study (*7*) betacyanins were extracted in water at 80 °C for 5 min, which readily caused coelution of pigment and proteins. Methanol (MeOH) can precipitate proteins and other insoluble products. In the present study, betacyanins were extracted with 80% MeOH and purified using the method described by Strack et al. (*3*) and Schliemann et al. (*18*). Frozen samples (10−450 g for different genotypes) were homogenized in a mortar and extracted with 80% MeOH. The homogenates were kept at 4−8 °C for 40−50 min with continuous stirring and were filtered through a Millipore filter (Millipore Corp., Bedford, MA) with a 0.2 μM nylon membrane under vacuum at 22 °C. The filtrates were centrifuged at 16000*g* for 15 min. The supernatant could be directly used for quantitative analysis by analytical HPLC or used for further purification of betacyanins. The extracts were concentrated under vacuum at 22 °C. The concentrate was transferred to a Sephadex LH-20−100 (Sigma Co., St. Louis, MO) column (100 × 2.5 cm i.d.) and eluted with ultrafiltered water. The final purification of the betacyanins was obtained by preparative HPLC. The purified samples were freeze-dried and used for qualitative analysis.

Table 1. Identification of Betacyanins in the Amaranthaceae

peak[a]	trivial name	definitive name[b]	t_R (min)	DAD-HPLC[c] I: acyl (nm)	II: λ_{max} (nm)	II:I ratio (abs)	$[M + H]^+$ [d]
1	amaranthine	betanidin 5-*O*-*β*-glucuronosylglucoside	10.71		536		727
1′	isoamaranthine	isobetanidin 5-*O*-*β*-glucuronosylglucoside	12.01		536		
2	betanin	betanidin 5-*O*-*β*-glucoside	14.77		538		551
2′	isobetanin	isobetanidin 5-*O*-*β*-glucoside	16.66		538		
3	Iresinin I	Betanidin 5-*O*-(6′-*O*-3-hydroxy-3-methyl-glutaryl)-*β*-glucuronosylglucoside	17.49	298	540	1:0.323	871
3′	isoiresinin I	isobetanidin 5-*O*-(6′-*O*-3-hydroxy-3-methyl-glutaryl)-*β*-glucuronosylglucoside	18.37	298	540	1:0.320	
4	celosianin I	betanidin 5-*O*-(2″-O-*E*-4-coumaroyl)-*β*-glucuronosylglucoside	19.90	306	546	1:0.526	872
4′	isocelosianin I	isobetanidin 5-*O*-(2″-O-*E*-4-coumaroyl)-*β*-glucuronosylglucoside	20.56	306	546	1:0.532	
5	celosianin II	betanidin 5-*O*-(2″-O-*E*-feruloyl)-*β*-glucuronosyl-glucoside	21.32	312	546	1:0.605	903
5′	isocelosianin II	isobetanidin 5-*O*-(2″-O-*E*-feruloyl)-*β*-glucuronosylglucoside	22.11	312	546	1:0.610	
6	gomphrenin I	betanidin 6-*O*-*β*-glucoside	32.83		540		551
6′	isogomphrenin I	isobetanidin 6-*O*-*β*-glucoside	35.19		540		
7	gomphrenin II	betanidin 6-*O*-(6′-*O*-*E*-4-coumaroyl)-*β*-glucoside	58.34	310	552	1:0.348	697
8	gomphrenin III	betanidin 6-*O*-(6′-*O*-*E*-feruloyl)-*β*-glucoside	59.10	322	552	1:0.340	727
7′	isogomphrenin II	isobetanidin 6-*O*-(6′-*O*-*E*-4-coumaroyl)-*β*-glucoside	61.58	310	552	1:0.383	
8′	isogomphrenin III	isoetanidin 6-*O*-(6′-*O*-*E*-feruloyl)-*β*-glucoside	63.59	324	552	1:0.392	

[a] The peak number is in order of elution (t_R) for the 16 betacyanins. " ′ " represents the betacyanin isomeric form. [b] -*β*-glucuronosyl-glucoside = -*β*-(1″,2′)-glucuronosyl-*β*-glucoside (*22*) = -(*β*-glucuronic acid)-*β*-glucoside (*2*) = -(*β*-D-glucosyluronic acid)-*β*-glucoside (*26*) = -(*β*-D-glucopyranosyluronic acid)-*β*-D-glucopyranoside (*14*). [c] The ratio of absorbance (abs) at the visible maximum (II:λ_{max}) to that at the UV maximum (I:acyl moiety). Ratio of absorbance at λ_{max} and 320 nm is 1:0.037−0.099 for nonacylated betacyanins. [d] Measured by ESI-MS.

HPLC Analysis. An HP 1100 series HPLC System (Hewlett-Packard) was used, consisting of a binary pump and a diode array detector (DAD), and operated at room temperature throughout the analysis. Data processing was performed using Hewlett-Packard HPLC 2D ChemStation software. Conditions for analytical HPLC were as follows: Nucleosil 100-C18 column (5 μm, 250 × 4 mm) with Nucleosil 5 C18 guard column (5 μm, 4 × 4 mm) (Agilent Technologies). Linear gradient elution was within 100 min from 0 to 100% solvent B (1.5% H_3PO_4, 20% acetic acid, and 25% acetonitrile in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) for *Gomphrena globosa* (Figure 1F) and within 30 min from 10 to 55% solvent B in solvent A for others (Figure 1A−E) at a flow rate of 1 mL/min with a 20-μL injection volume and detection at 540 nm. Conditions for preparative HPLC were as follows: Zorbax SB-C18 column (5 μm, 250 × 9.4 mm) with SB-C8 guard column (5 μm, 15 × 9.4 mm) (Agilent Technologies); linear gradient within 60 min from 20% solvent B (80% aqueous MeOH) in solvent A (1% aqueous formic acid) to 40% B in A + B; 4 mL/min flow rate; 0.1 mL injection volume; detection at 540 nm.

Mass Spectrometry of Betacyanins. Electrospray ionization mass spectrometry (ESI-MS and ESI-MS-MS) was performed on a quadrupole ion-trap mass spectrometer (Finnigan MAT LCQ, San Jose, CA) equipped with an electrospray ionization source. Betacyanin samples (concentrations of 0.6−1.0 mg/mL) were dissolved in methanol (∼1:10) and 0.1 M ammonium acetate (1:1) as described by Heuer et al. (*16*) and then analyzed by ESI-MS and ESI-MS-MS at a flow rate of 5 μL/min under an ion spray voltage of 4.5 kV, a capillary temperature of 200 °C, a capillary voltage of 3−8 V, a tube lens offset of 20−40 V, and a collision energy of 25−40 eV.

Quantitative Determination of Betacyanins. Individual betacyanin composition (percent) was measured by analytical HPLC and was expressed as percentage of peak area according to the method of Drdak et al. (*19*) and Cai et al. (*7*). Before HPLC analysis, a portion of the supernatant of betacyanin extracts was frozen at −18 °C overnight, then thawed, and recentrifuged to remove impurities (protein, etc.) to reduce interference with chromatographic determination. Total betacyanin contents (milligrams per gram of fresh weight) were determined on a Spectronic Genesys 5 spectrophotometer (Milton Roy, NY). The total contents of amaranthine-type betacyanins (betanin- or gomphrenin-type betacyanins) were calculated using the formula reported by Cai et al. (*7*) with little modification (delete 10^2 in the original formula) and expressed as milligrams of amaranthine (betanin or gomphrenin I) per gram of fresh tissue. Their molecular weight (M_w) values were 726.6 (amaranthine), 550 (betanin), and 550 (gomphrenin I), and their molar absorptivity (ϵ) values were 5.66×10^4 [amaranthine, $E^{1\%}_{1cm}$ (536 nm) = 779; *20*], 6.16×10^4 [betanin, $E^{1\%}_{1cm}$ (536 nm) = 1120; *21*], and 5.06×10^4 [gomphrenin I, $E^{1\%}_{1cm}$ (540 nm) = 920; *15*]. Duplicate measurements were performed for each genotype.

Alkaline Hydrolysis of Betacyanins. Pure acylated betacyanins were hydrolyzed, as described by Minale et al. (*15*) and Strack et al. (*3*), in a screw-cap tube with 0.2 N NaOH in 50% methanol under vacuum at room temperature for 3 h and then acidified with 2 N HCl to pH 2.8−3.5 to restore the original red-violet color. The hydrolysates were purified on a Sephadex LH-20−100 column (30 × 2.5 cm i.d.) and then directly detected by HPLC. The liberated acyl compounds (*p*-coumaric acid, ferulic acid, etc.) were identified by chromatographic comparison with pure standards (Sigma Co., St. Louis, MO) under the analytical conditions described by Schliemann et al. (*18*) and Giusti and Wrolstand (*13*).

RESULTS AND DISCUSSION

Identification of Simple and Acylated Betacyanins. Identification of the most common pigments in the order Caryophyllales can be ascertained by cochromatography with authentic samples and by comparison with literature data (*2*, *3*). Several typical HPLC profiles (Figure 1) of methanolic extracts from different plant materials in the Amaranthaceae display 16 distinct peaks altogether at a detection wavelength of 540 nm. Identification and structures of eight betacyanins and their stereoisomers (C-15 epimers) isolated in this family are summarized in Table 1 and Figure 2. The number of the peaks in Table 1 and Figure 1 coincides with the number of betacyanins in Figure 2.

The major peaks (**1** and **1′**) were readily identified by cochromatography with the reference extracts of amaranthine (betanidin 5-*O*-*β*-glucuronosylglucoside) and isoamaranthine (C-15 epimer) from *A. tricolor* leaves (*1*, *6*, *7*). The peaks **2** and **2′** were also easily identified by cochromatography with the authentic samples of beta-

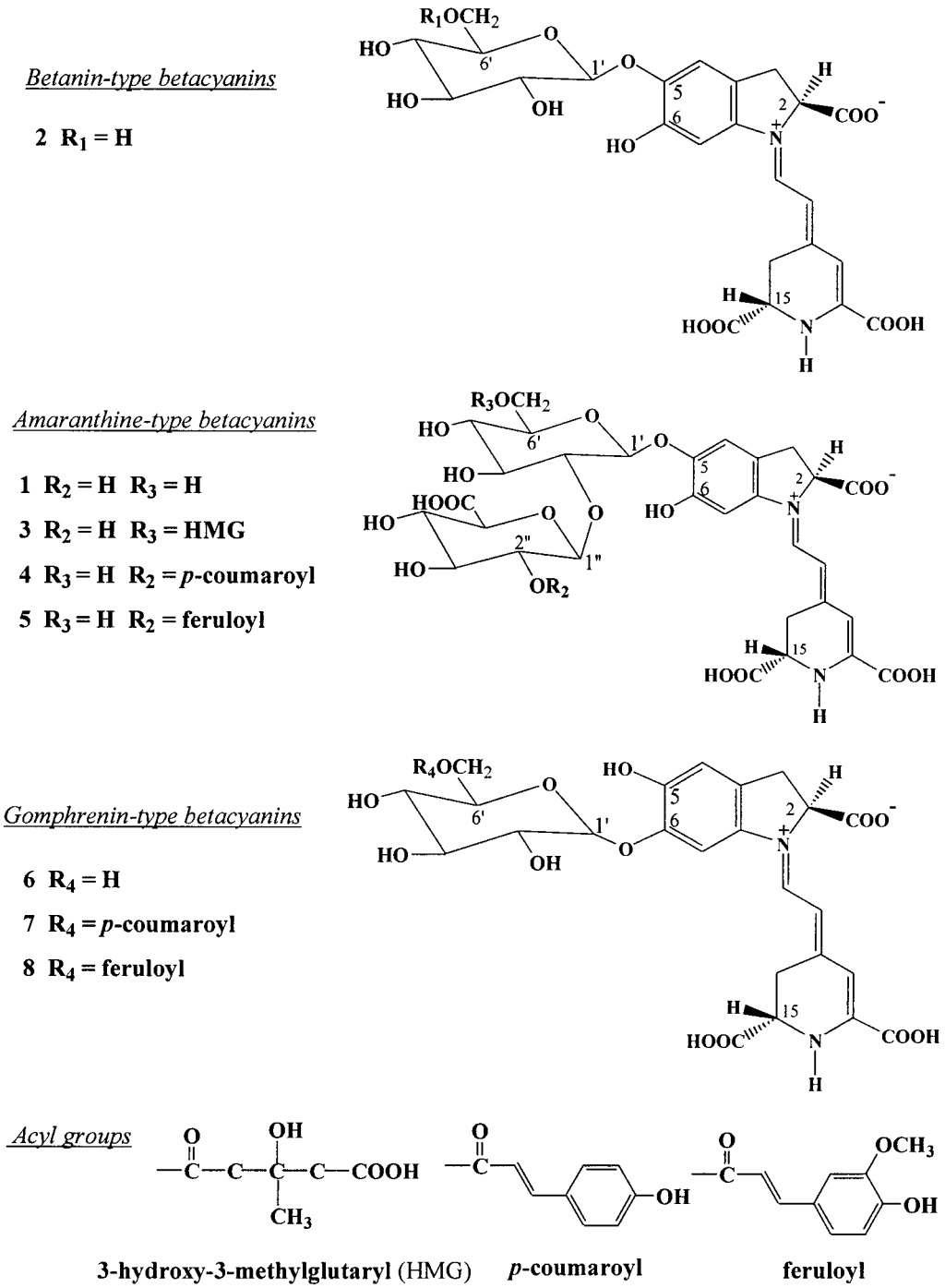

Figure 2. Structures of betacyanins in the Amaranthaceae: betanin (**2**); amaranthine (**1**); iresinin I (**3**); celosianin I (**4**); celosianin II (**5**); gomphrenin I (**6**); gomphrenin II (**7**); gomphrenin III (**8**). Their isomeric forms (**1′**, **2′**, **3′**, **4′**, **5′**, **6′**, **7′**, and **8′**) are the C-15 epimers of corresponding betacyanins (not drawn).

nin (betanidin 5-*O*-β-glucoside) and isobetanin (C-15 epimer) from red beet pigments (No. 3600, Warner-Jenkinson Co., Inc., St. Louis, MO). Table 1 and Figure 2 clearly show that these four pigments (**1**, **1′**, **2**, and **2′**) were simple betacyanins without any acyl groups.

According to the UV−vis spectra, chromatographic profiles, ion spray mass spectra, and alkaline hydrolysis, peaks **3**−**8′** (except for peaks **6** and **6′**) were identified as acylated betacyanins by comparison with literature data (*2*, *3*). Peaks **3**−**5**) were assumed to be iresinin I, celosianin I, and celosianin II, respectively. They were the amaranthine-type (substituted at C-5 of betanidin) betacyanins with acyl groups (3-hydroxy-3-methylglutaryl, *p*-coumaroyl, and feruloyl) (Figure 2). Minale et al. (*14*), Strack et al. (*22*), and Bokern and Strack (*23*) reported similar results and detected the same acylated betacyanins in other plant materials. The presence of their isobetacyanins (peaks **3′**, **4′**, and **5′**) was easily confirmed by treatment of the extracts with an aqueous citric acid solution as described by Piattelli and Minale (*1*). The six peaks (**6**, **6′**, **7**, **7′**, **8**, and **8′**) isolated from *Gomphrena globosa* (Figure 1F) completely matched those detected and identified by Heuer et al. (*16*) at the same HPLC conditions. They were gomphrenin-type betacyanins substituted at C-6 of betanidin (Figure 2) (*3*). They were gomphrenin I (peak **6**) (betanidin 6-*O*-β-glucoside, nonacylated betacyanins) and their acylated forms with *p*-coumaroyl and feruloyl, that is, gomphre-

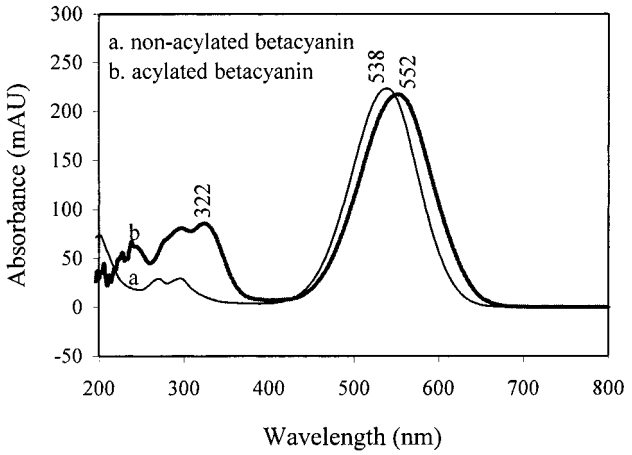

Figure 3. UV−vis spectra of nonacylated betacyanin (a, amaranthine) and acylated betacyanin (b, gomphrenin III).

nin II (**7**) and gomphrenin III (**8**). Their isomeric forms were isogomphrenin I (**6′**), isogomphrenin II (**7′**), and isogomphrenin III (**8′**). These acylated betacyanins could also be indirectly identified by alkaline hydrolysis treatment (*3*, *18*). When treated with 0.2 N NaOH in 50% aqueous methanol, their hydrolysates were detected by HPLC. The results confirmed that peaks **3**−**5** were in part deacylated into amaranthine (**1**), whereas peaks **7** and **8** were in part deacylated into gomphrenin I (**6**). The liberated acyl groups (ferulic acid, *p*-coumaric acid, and 3-hydroxy-3-methylglutaric acid) were readily identified by comparison of retention time and spectral data using pure standard samples.

UV−vis spectra by DAD-HPLC (Table 1) showed that the betacyanins isolated exhibited maxima (λ_{max}) between 536 and 552 nm in the visible region. Acylated betacyanins displayed an additional absorption in the UV region of ∼300−330 nm, where the absorption of nonacylated betacyanins was weak (Figure 3). Moreover, it was observed that acylated betacyanins, like anthocyanins acylated with aromatic acids, mainly hydroxycinnamic acids, exhibited a marked bathochromic shift. The λ_{max} values in the visible region were 540−552 nm for acylated betacyanins (**3**, **3′**, **4**, **4′**, **5**, **5′**, **7**, **7′**, **8**, and **8′**) but 536−540 nm for nonacylated betacyanins (**1**, **1′**, **2**, **2′**, **6**, and **6′**). The results were similar to those reported by Heuer et al. (*16*), Jackman and Smith (*2*), and Schliemann and Strack (*17*). However, the λ_{max} values of gomphrenin-type betacyanins in our study were slightly different from those (540, 542, and 549 nm for gomphrenins I, II, and III, respectively) published by Minale et al. (*15*). The number of acyl residues in betacyanins, as in anthocyanins, could be estimated from the ratio of absorption at the visible maximum to that at the UV maximum (*3*, *16*). The ratio of absorption at the visible maximum (betacyanin glycoside moiety) to that at the UV maximum (acyl moiety) was 1:0.32−0.61 for all acylated betacyanins in this study. This suggested that they were mono-acyl-betacyanins.

The chromatographic behavior of the betacyanins further supported our identification. HPLC elution order (retention time t_R, Table 1 and Figure 1) revealed that (i) the retention of gomphrenin-type betacyanins (**6**, **6′**, **7**, **7′**, **8**, and **8′**) (6-*O*-glucosides) obviously exceeded that of amaranthine- (**1**, **1′**, **3**, **3′**, **4**, **4′**, **5**, and **5′**) or betanin-type (**2** and **2′**) betacyanins (5-*O*-glucuronosylglucosides or 5-*O*-glucosides) because of their structural difference at C-5 and C-6; (ii) acylated betacyanins (**3**−**5′** and **7**−**8′**) had a longer retention time than nonacylated betacyanins (**1**-**4′**, **6**, and **6′**) because acyl groups increased the retention of pigments; (iii) the retention of betacyanins decreased with the increase of glycosyl substitution;

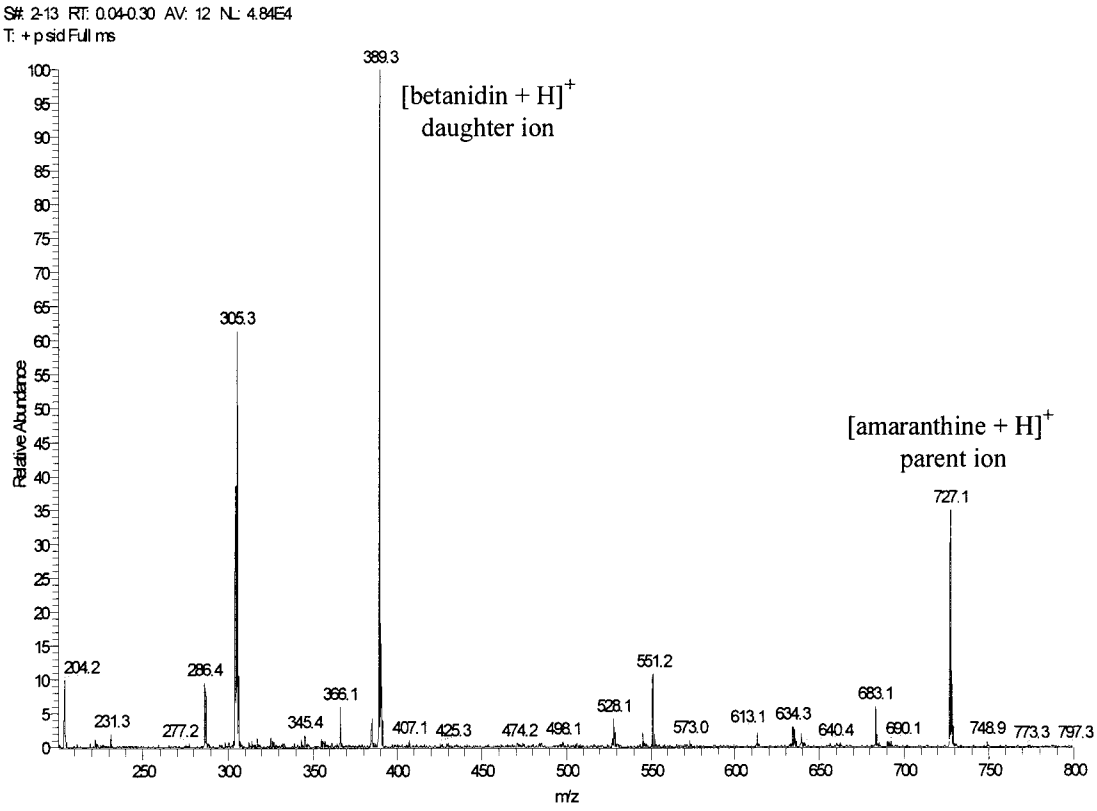

Figure 4. Representative mass spectrum of the predominant betacyanin in the Amaranthaceae (i.e., amaranthine separated from *A. cruentus* L.): daughter ion [betanidin + H]$^+$ at *m*/*z* 389 and parent ion [M + H] at *m*/*z* 727.

Table 2. Quantification and Distribution of Simple (Nonacylated) and Acylated Betacyanins in the Amaranthaceae

genus and species[a]	genotype name	origin	plant part[b]	individual betacyanin composition[c] (%) amaranthine	isoamaranthine	betanin	isobetanin	celosianin I	isocelosianin I	celosianin II	isocelosianin II	total pigment content (mg/g)[d]
Achyranthes												
A. aspera L.	Am10	China	sd	94.7	2.6	1.7						0.11
A. bidentata F. rubra Ho.	Am11	China	sd	95.7	2.2	1.3	0.3					0.28
Alternanthera												
A. ficoidea (L.) R. Br.	Am06	China	lv	95.1	2.8	1.2	0.5					0.27
A. bettzickiana Nichols.	Am07	China	lv	92.0	1.6	1.2	0.5	1.3		2.9	tr	0.35
Amaranthus												
A. caudatus L.	Ca1657	U.K.	sd	95.3	2.8	0.7						0.73
	San119	China	inf	97.7	2.0							1.22
A. acutilobus L.	Ac001	unknown	sd	96.2	3.0							0.15
A. albus L.	Al002	Spain	sd	94.1	5.4							0.57
A. australis L.	Au002	U.S.	sd	88.1	8.4	0.6		0.6		1.8	0.2	0.35
A. blitoides S. Wates.	Bl001	Hungary	st	95.7	tr	3.1						0.12
A. cannabinus L.	Cn001	U.S.	sd	96.8	1.2	1.5						0.12
A. cruentus L.	Cr071	U.S.	sd	97.0	1.6	1.0	0.2					1.36
	Cr072	India	lv	96.1	1.7	1.8						1.27
A. deflexus L.	De002	France	sd	97.4	1.1	0.8						0.63
A. dubius L.	Du004	Zimbabwe	sd	96.2	2.3	tr						0.09
A. fimbriatus L.	Fm001	Mexico	sd	94.9	1.7	2.9						0.37
A. graecizans L.	Gr001	U.S.	sd	93.3	tr	tr						0.08
A. hybridus L.	Hr008	Zimbabwe	sd	96.1	1.2	2.5						0.76
A. hypochondriacus L.	Hy041	U.S.	inf	95.5	2.0	0.8	tr					1.08
A. lividus L.	Lv003	India	sd	97.0	1.9	0.5						0.45
A. mangostanus L.	Xian C.	China	sd	95.6	2.2	1.3						0.36
A. palmeri L.	Pa002	U.S.	st	96.4	tr					1.9		0.24
A. paniculatus L.	Tibet Y.	China	sd	97.3	1.1	0.8						0.87
A. powellii L.	Pl002	Rwanda	sd	95.0	1.1	2.7	0.9					0.34
A. pumilus L.	Pu001	U.S.	sd	97.3	1.3	1.4						0.35
A. quitensis L.	Qu002	Bolivia	sd	94.8	2.0	0.6						0.58
A. retroflexus L.	Re003	Turkey	sd	96.6	tr	2.6						0.50
A. rudis L.	Ru001	U.S.	sd	97.1	1.0	1.0						0.11
A. spinosus L.	Sn002	Taiwan	sd	94.9	2.2	1.0						0.26
A. standleyanus L.	St001	Argentina	sd	94.3	0.8	tr						0.08
A. tricolor L.	Tr011	China	sd	95.6	4.1							1.05
	Beijing R.	China	lv	93.5	4.6	1.2	tr					1.28
A. viridus L.	Vd002	Maldives	st	90.7	2.7	tr		tr		1.8		0.18
Aerva												
A. sanguinolenta (L.) Bl.	Am09	China	inf	93.5	1.8			1.5		2.6		0.15
*Celosia**												
C. argentea L.	Am01	China	inf	92.3	3.1	0.9		tr				0.44
C. cristata L.	Am02	Australia	inf	81.3	12.1	0.6		1.2		3.1	0.4	0.83
C. cristata L. var. (violet)	Am03	China	inf	51.5	8.3			6.1	2.0	28.8	2.9	1.17
Cyathula												
C. capitata Moq.	Am08	China	rt	88.3	3.6	1.4	tr	1.8		6.7	1.1	0.31
mean				91.5[e]	2.8[e]	1.4	0.5	2.1	2.0	6.2	1.2	0.54[e]

genus and species[a]	genotype name	origin	plant part[b]	individual betacyanin composition (%)[c] amaranthine	isoamaranthine	iresinin I	isoiresinin I	gomphrenin I	isogomphrenin I	gomphrenin II	isogomphrenin II	gomphrenin III	isogomphrenin III	total pigment content (mg/g)[d]
*Gomphrena**														
G. globosa L.	Am04	India	inf	tr				16.9	8.8	11.1	3.5	40.8	13.0	1.30
*Iresine**														
I. herbstii Hook. F. ex Lindl.	Am05	China	lv	17.8	0.8	66.5	13.1							0.75
mean				91.5[e]	2.8[e]	66.5	13.1	16.9	8.8	11.1	3.5	40.8	13.0	0.54[e]

[a] An asterisk indicates genera that were field-grown in normal season; other genera were planted in a greenhouse in late winter and early spring. [b] Abbreviations: sd, seedlings; lv, leaves; inf, inflorescences; st, stems; rt, roots. [c] Individual betacyanin composition was expressed as percentage of peak area determined by HPLC. tr, trace amount (small peak) <0.1%. [d] Total betacyanin content was measured by a spectrophotometric method and calculated as milligrams of amaranthine (betanin or gomphrenin I) per gram of fresh material, means of two replicates. [e] Overall mean of 40 genotypes.

thus, amaranthine-type betacyanins with two sugar units attached had a shorter retention time than the betacyanins with only one sugar unit attached (betanin- or gomphrenin-type); and (iv) because the configuration of C-15 isomeric forms for betacyanins allowed greater interaction with the stationary phase, isobetacyanins were retained slightly longer than their parents. Not only did the results agree well with those predicted by reversed-phase chromatography principles, they were also similar to earlier results reported by Piattelli and Minale (*1*), Heuer et al. (*16*), Jackman and Smith (*2*), and so on.

A more reliable and direct confirmation of the betacyanin molecular composition came from the electrospray mass spectra that gave the expected protonated molecular ions and various fragment ions. This mass spectrometric technique has been successfully used to confirm the molecular mass of betacyanins and provides important structural information (*16*, *18*, *22*). Table 1 shows that the molecular weights of the betacyanins (**1**−**8**) measured by ESI-MS agreed quite well with the calculated masses for monoisotopic protonated molecular ions. The masses of the isomeric betacyanins (**1′**−**8′**) were not determined by ESI-MS in this study

because the isobetacyanins possessed the same masses as the betacyanins and could not be distinguished from the betacyanins in the mass spectra. ESI-MS-MS allowed acquisition of daughter ion scans of the protonated molecular ions $[M + H]^+$ (Table 1) and yielded in all cases the fragment ion [betanidin + H]$^+$ at mass/charge (*m*/*z*) 389, which indicated that these $[M + H]^+$ ions were derived from the same aglycon betanidin. Figure 4 displays one typical mass spectrum of the predominant betacyanin (i.e., amaranthine) in the Amaranthaceae, showing daughter ion [betanidin + H]$^+$ at *m*/*z* 389 and parent ion $[M + H]^+$ at *m*/*z* 727. The fragment at *m*/*z* 551 with low intensity was [betanin + H]$^+$; the collision of the parent ion (*m*/*z* 727) lost one glucose moiety. The mass spectra of other fragments (e.g., *m*/*z* 551) obtained by ESI-MS-MS were also helpful for the structure elucidation of betacyanins (the mass spectra of other betacyanins were omitted in this paper).

Distribution and Quantification of Simple and Acylated Betacyanins. Distribution and quantification of the betacyanins in most of the species and genotypes examined in the Amaranthaceae have not been described previously. The quantitative analysis results (Table 2) revealed that simple (nonacylated) betacyanins, such as amaranthine, betanin, and their isomeric compounds, were widespread among all genera of the Amaranthaceae. Among 40 genotypes of 37 species in 8 genera, the average amaranthine and isoamaranthine compositions, expressed as percentage of peak area, were 91.5 and 2.8% of the total area, indicating they were predominant betacyanins in this family. Of these, 27 genotypes contained low amounts of betanin (1.4%) and isobetanin (0.5%) that could be considered as the predominant betacyanins in the family Chenopodiaceae. To a certain extent, the findings were in accordance with previous investigations of 16 species in the Amaranthaceae by polyamide chromatography (*1*). However, our previous work on the pigments from 21 genotypes of 7 species in the genus *Amaranthus* identified them as only homogeneous amaranthine and isoamaranthine (*7*). Other researchers (*6*, *24*) also did not identify betanin and isobetanin in this genus. This was probably due to the use of more sensitive analytical methods and extraction methods (methanol) and different chromatographic conditions in this study.

Table 2 also shows that acylated betacyanins were distributed in seven of eight genera examined in the Amaranthaceae except *Achyranthes*. *Iresine herbstii* Hook. F. ex Lindl. contained the highest proportion (79.6% of total peak area) of acylated betacyanin compositions (iresinin I and isoiresinin I). *G. globosa* L. possessed quite a high level (68.4%) of acylated betacyanins (gomphrenins I and II and their C-15 epimers). *Celosia cristata* L. (violet) contained 39.8% acylated betacyanins (celosianins I and II and their C-15 epimers). Although celosianins I and II and their isomers were first isolated in the present study from six wild species of four genera (*Amaranthus*, *Alternanthera*, *Aerva*, and *Cyathula*), their amount was quite low (overall average of 4.0%) compared to those of above three species. This possibly resulted from real differences in betacyanin biosynthesis among the various species in the Amaranthaceae, but some difference might be due to different planting conditions. The former were planted in a greenhouse (lack of sunlight) in late winter and early spring, whereas the latter (the three species with high levels of acylated betacyanins) were grown in a field in the normal season. Biosynthesis of the betacyanins was usually affected by sunlight, temperature, etc. (*20*, *25*, *26*). Bokern and Strack (*23*) reported acylated betacyanins could be formed via hydroxycinnamic acid-glucoside-dependent acyltransferases. Strack et al. (*27*) also found the acyltransferase in *Amaranthus cruentus*. This type of acyl formation might be widespread among betacyanin-containing plants. Additionally, according to statistical analysis of the data in Table 2, five cultivated species (*I. herbstii* Hook. F. ex Lindl., *G. globosa*, and three *Celosia* species) contained many more acylated betacyanins (overall average of 38.5%) than six wild (weedy) species (4.0% as described above).

Total betacyanin content ranged from 0.08 to 1.36 mg/g of fresh weight (Table 2), showing extensive variation in betacyanin content among and within species in the Amaranthaceae. The highest betacyanin content was in *A. cruentus* (1.36 mg/g) and *G. globosa* (1.30 mg/g). It was also found that cultivated species (mean = 0.94 mg/g), such as *A. cruentus*, *Amaranthus tricolor*, *Amaranthus caudatus*, *C. cristata* (violet), and *G. globosa*, possessed much higher total betacyanin content than wild (weedy) species (mean = 0.27 mg/g), consistent with our previous results (*7*). Nevertheless, in this study, the average betacyanin content of 40 genotypes was only 0.54 mg/g, lower than our previously reported data (mean = 0.91 mg/g for 21 *Amaranthus* genotypes grown in a normal season). The reasons probably include the following: (1) most genotypes in this study were planted in a greenhouse; (2) most genotypes were from wild species; and (3) betacyanin extraction with methanol in this study was not as effective as water extraction used previously.

The effect of acyl groups on the color intensity and stability of betacyanin pigments is not clear. Further research to elucidate the stability difference between simple and acylated betacyanins is required. Our previous results (*9*) showed there were significant genotypic differences in pigment stability among *Amaranthus* species. The evidence for the acylated betacyanins detected in the present study is not sufficient to explain the causes of the stability differences. The abundance of acylated betacyanins in some cultivated species may offer a new source of more stable red-violet pigments for the food industry and also provide a potential alternative source of betalains similar to those extracted from red beets.

ACKNOWLEDGMENT

We thank David Brenner (Department of Agronomy, Iowa State University) and Prof. Hongliang Sun (Chinese Academy of Agricultural Science, Beijing, China) for providing genetic resources and Ronghua Huang (Hubei Academy of Agricultural Sciences, Wuhan, China) for field assistance.

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Received for review August 1, 2000. Revised manuscript received January 24, 2001. Accepted January 26, 2001. The financial support of the Hong Kong Research Grants Council is acknowledged.

JF000963H