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Profiles of Betacyanins in Epidermal Layers of Grafted and Light-Stressed Cacti Studied by LC-DAD-ESI-MS/MS

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Profiles of betacyanins present in light-stressed stems of different cactus species and nonstressed but genetically aberrated red pigmented cacti of *Gymnocalycium mihanovichii* cv. 'Hibotan' Britton & Rose, which are known as grafted cacti, were compared. The identities of all the pigments in the cacti were characterized for the first time. The identification of acylated and nonacylated betacyanins was performed by means of mass spectrometry and UV−vis diode array detection coupled to high-performance liquid chromatography. The most indicative pigments of the stressed cacti, 5″-*O*-*E*-feruloyl-2′-*O*-β-apiosyl-betanin and 5″-*O*-*E*-sinapoyl-2′-*O*-β-apiosyl-betanin, as well as their diasteromers, were the prevailing betacyanins in *Hylocereus polyrhizus*, *Epiphyllum phyllanthus*, and *Rhipsalis rhombea*. Stressed *Rhipsalis regnellii* stems contained the sinapoylated betacyanins accompanied only by traces of the feruloylated derivatives. In addition, high contents of 2′-*O*-apiosyl-betanin were frequently observed in the samples with the highest concentration found in stressed *Schlumbergera* × *buckleyi* (T. Moore) Tjaden. These pigments were also detected, but at low levels, in the Hibotan pink, red, and violet scions, which were not light-stressed. In the Hibotan scions, the most abundant were the polar betacyanins: betanidin 5-*O*-β-sophoroside and betanin. In most of the stressed samples, betanin was present at relatively low levels.

KEYWORDS: Abiotic stress; antioxidants; betacyanins; betanin; phyllocactin; mammillarinin; HPLC-DAD-ESI-MS/MS

INTRODUCTION

Betalains (betacyanins and betaxanthins) constitute a class of secondary metabolites found in species of the Caryophyllales, with the exception of members of the families Caryophyllaceae and Molluginaceae, accumulating anthocyanins (*1*). Interest in betalains has grown since their antiradical activity was characterized (*2*−*6*), and they are widely used as additives for foods and drugs as well as cosmetic products because of their natural colorant properties and absence of toxicity (*7*). Early investigations of betacyanins resulted in publication of a series of their chemical structures present in many plants; however, due to the lack of appropriate techniques, many of these red-violet pigments remained unknown. Recent extensive studies revealed the presence of new betalains in cactus flowers and fruits (*8*−*12*); however, no relevant investigations were performed on betalains indicating stress in epidermal cactus stem layers.

Abiotic stress can be induced by numerous factors promoting synthesis of secondary metabolites in plants (*13*, *14*). The response to stress can be indicated by a production of pigments on plant leaves or stems. This is frequently noted for such pigments as anthocyanins or betalains; however, little is known, especially for betalains, about the function of the pigments in the vegetative organs (*15*).

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The biosynthesis of betalains is regulated, for example, by light, temperature, or the presence of cytokinins and abscisic acid (*16*, *17*). Recently, oxidative stress leading to betacyanin production in leaves of halophyte *Suaeda salsa* L. was induced by watering roots with H_2O_2 (*18*). Interestingly, further research on *S. salsa* L. indicated the negative effect of light on betacyanin accumulation in the plant, which may be explained by the activity of tyrosinase (degraded or deactivated by light) (*19*).

Betacyanins (**Figure 1**) were most probably scavengers of reactive oxygen species in *Beta vulgaris* L. leaves during an oxidative burst induced by bacterial infection and wounding (*20*). In turn, generation of reactive oxygen species appeared to be a key event that preceded and activated betacyanin accumulation (*20*). The accumulation of betacyanins upon light irradiation was demonstrated in another salt-tolerant halophyte, *Mesembryanthemum crystallinum* L. (*21*, *22*).

Biotic and abiotic stress can be used to stimulate betalain formation in plant cell cultures (*23*−*25*), which can be valuable sources of these health-promoting compounds. This can result in reducing the process time to reach high pigment concentrations. Photoirradiation could be applied for enhancement of the pigment content and structural variety in cell cultures (e.g., of *B. vulgaris* L.); however, knowledge about the influence of photoinduced stress (e.g., by the daylight irradiation) or any stress on structural composition of betalain pigments in plants is very scarce (*22*).

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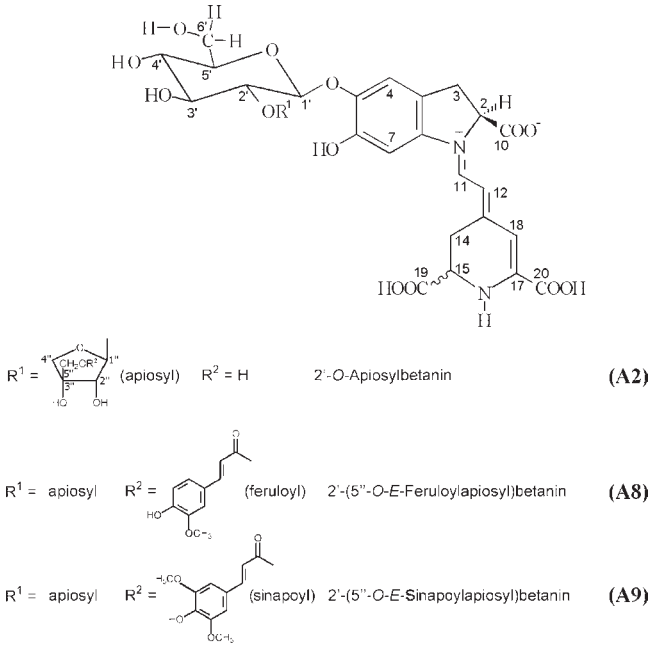

Figure 1. Chemical structures of basic apiosylated betacyanin (**A2**) as well as feruloylated (**A8**) and sinapoylated (**A9**) derivatives present in stressed cactus samples.

In contrast to the stressed cacti, betacyanins are also present in epidermal layers of nonstressed but genetically aberrated cacti of *Gymnocalycium mihanovichii* cv. 'Hibotan' Britton & Rose, which together with other varieties are known as grafted cacti characterized by a palette of various colors ranging from yellow through orange, red, pink, violet to dark violet, brown, and black (*26*). These variations are a result of the absence of part or all of the chlorophyll in the plant and the presence of a combination of other pigments (betacyanins and carotenoids) in normal concentration, the so-called schizochromism phenomenon. These plants cannot survive themselves because of the lack of chlorophyll, or part thereof; therefore, they are grafted onto stocks of other green cacti. The Hibotan cacti originated in Japan in the 1940s and are commercially well-known.

This study was undertaken to determine the chromatographic profiles of betacyanins present in light-stressed cactus stems and in nonstressed grafted cacti analyzed by LC-DAD-ESI-MS/MS. From Hibotan cacti, the red, pink, and violet varieties, frequently grown and sold in European countries, were the subjects of our study. Nothing except the total pigment concentrations in Hibotan (*26*) was known about the betacyanin composition in the epiderm of the cacti in which most of the pigments accumulated, and a comparison between chromatographic profiles of some stressed and nonstressed cacti was of interest.

MATERIALS AND METHODS

Reagents. Formic acid, trifluoroacetic acid (TFA), HPLC grade acetonitrile, and HPLC grade water were obtained from Merck (Darmstadt, Germany).

Plant Material. Stems of six stressed cactus species prepared for the study were obtained from the Botanical Garden of Jagiellonian University Institute of Botany (Cracow, Poland) during the seasons of 2005−2007: *Rhipsalis rhombea* (Salm-Dyck) Pfeiff., *Rhipsalis houlentianum* Lem., *Rhipsalis regnellii* G.A. Lindb., *Epiphyllum phyllanthus* (L.) Haw., *Selenicereus megalanthus* (K. Schum. ex Vaupel) Moran, and *Schlumbergera* × *buckleyi* (T. Moore) Tjaden (Christmas cactus). *Hylocereus polyrhizus* (FAC Weber) Britton and Rose light-stressed stems were collected in orchards of the Department of Life Sciences (Ben Gurion University of the Negev, Beer-Sheva, Israel) in the season of 2005. All of the plants were exposed to sunlight for at least a 1 year period until violet pigments were accumulated in the stem epiderm. Three varieties of red, pink, and violet Hibotan cacti (*G. mihanovichii* cv. 'Hibotan' Britton & Rose) were obtained from the private collection of Paweł Nalaskowski (Żary, Poland).

Pigment Extraction. Typically, 20 g of stressed stems and peeled grafted cacti was separately extracted three times with 100 mL of 80% of aqueous MeOH in an ice-cooled blender and subsequently filtered through a 0.2 μm i.d. pore size filter (Millipore, Bedford, MA). The extracts were concentrated using a rotary evaporator under reduced pressure at 25 °C and freeze-dried. For the co-injection experiments the extracts of *H. polyrhizus* and *Mammillaria coronata* Scheidw. fruits and *Schlumbergera* × *buckleyi* flowers from the previous studies (*11*, *12*) as well as *M. crystallinum* L. (*21*, *22*) were used.

Pigment Purification. For isolation, the pigment extracts were chromatographically concentrated by solid phase extraction on C18 cartridges (Merck) according to the procedure of Stintzing et al. (*27*). After rinsing with water, the betalain fraction was eluted with acidified methanol (methanol/TFA acidified water at pH 2, 95:5, v/v). The eluates were pooled and concentrated using a rotary evaporator under reduced pressure at 25 °C, freeze-dried, and dissolved in water before the chromatographic analysis.

Total Betacyanin Quantification. Quantification of betacyanins was performed according to a spectrophotometric method of Nilsson (*28*) using a UV−vis spectrophotometer Shimadzu 1650 PC (Shimadzu Corp., Kyoto, Japan). The determination of betacyanin concentration was calculated in terms of betanin on the basis of its absorptivity value $A^{1\%}$ 1120.

LC-DAD-ESI-MS/MS Analysis. A Gynkotek HPLC system with UVD340U, Gynkotek HPLC pump series LPG-3400A, and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used for the chromatographic analysis. For the data acquisition, the software package Chromeleon 6.0 (Gynkotek Separations) was used. For the UV−vis spectral acquisition the detection was performed in the diode array detection (DAD) mode. For the separation of betacyanins a Luna C18(2) column, 250 × 3 mm i.d., protected by a guard column (Phenomenex, Torrance, CA) was used. For the separation of the analytes the following gradient system was used: 95% A with 5% B at 0 min; gradient to 80% A with 20% B at 35 min (solvent A, 2% HCOOH; solvent B, acetonitrile). The injection volume was 10 μL, and the flow rate was 0.5 mL/min. The column was thermostated at 35 °C. Detection was performed in the UV−vis detector at λ = 538, 505, and 480 nm and in the DAD mode as well as by mass spectrometry on a ThermoFinnigan LCQ Advantage (electrospray voltage, 4.5 kV; capillary, 250 °C; sheath gas, N_2). The MS was controlled, and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA). The relative collision energies for the CID experiments were set at 30% (according to a relative energy scale). Helium was used to improve trapping efficiency and as the collision gas for the CID experiments.

RESULTS AND DISCUSSION

Betacyanins in Stressed Samples. The presence of betacyanins in stem epiderm of stressed cacti was predicted by observation of the characteristic violet-brown color apperaring on green parts of the plants. Betacyanins were also detected in epidermal parts of *Phytolacca americana* stems (*29*). Our preliminary study performed on *H. polyrhizus* light-stressed stems confirmed the presence of the pigments; therefore, our goal was to characterize betacyanins in specific cactus species that had been submitted to a few-year sunlight stress (**Figure 2**). The identified pigments are listed in **Table 1**, and their concentration profiles are shown in **Table 2**.

The total concentrations of betacyanins in the analyzed samples expressed in betanin equivalents were in the range of 3.9−52.4 μg/g, and the highest concentration was found in stressed *Schl.* × *buckleyi* stems (**Table 2**). Further study confirmed that in most of the samples betanin was not the prevailing betacyanin.

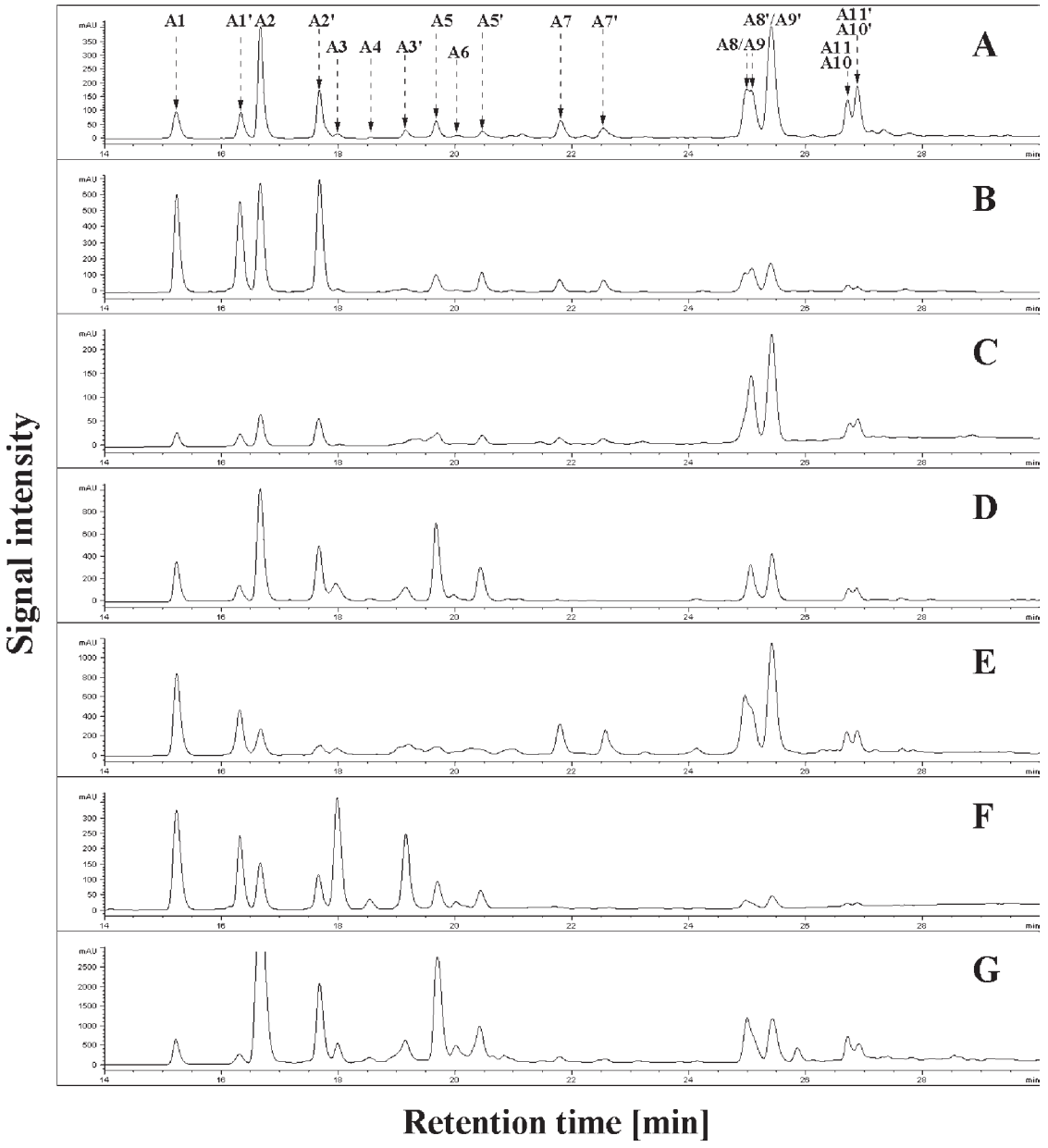

Figure 2. HPLC elution profiles of betacyanins (λ = 538 nm) in stressed cacti: (**A**) *Hylocereus polyrhizus*; (**B**) *Selenicereus megalanthus*; (**C**) *Epiphyllum phyllanthus*; (**D**) *Rhipsalis regnellii*; (**E**) *Rhipsalis rhombea*; (**F**) *Rhipsalis houlentianum*; (**G**) *Schlumbergera* × *buckleyi*.

The LC-DAD-MS/MS analyses revealed interesting pigment patterns formed primarily by **A2** and acylated betacyanins **A7**−**A9**. Pigments **A8**/**A8′**−**A9**/**A9′** exhibited ratios of absorbances at ca. 550 and 330 nm (ca. 1:0.5), indicating the presence of one hydroxycinnamoyl acylating moiety in each compound. Their protonated molecular ions ($[M + H]^+$) were found at m/z 859 and 889, respectively. The mass differences between **A8** (m/z 859), **A9** (m/z 889), and **A2** (m/z 683) suggested the presence of feruloyl ($\Delta m/z$ 859 − 683 = 176) and sinapoyl ($\Delta m/z$ 889 − 683 = 206) moieties in **A8** and **A9**, respectively. Cochromatographic experiments on **A8**/**A8′**−**A9**/**A9′** with the pigments derived from *H. ocamponis* (*11*) and *P. americana* (*30*) confirmed the presence of 5″-*O*-*E*-feruloyl-2′-*O*-*β*-apiosyl-betanin/isobetanin **A8**/**A8′** and 5″-*O*-*E*-sinapoyl-2′-*O*-*β*-apiosyl-betanin/isobetanin **A9**/**A9′**. These pigments were present at the highest proportion in *H. polyrhizus*, *E. phyllanthus*, and *R. rhombea* (**Table 2**; **Figure 2**). In contrast to most of the polar betacyanins, the isoforms **A8′**−**A9′** were the dominating diastereomers. It is also worth noting that **A9**/**A9′** was accompanied only by traces of A8/**A8′** in *R. regnellii*, which was a first case observed in contrast to the other plant samples (*11*, *30*).

The LC-DAD-MS/MS determination of **A7**/**A7′** present in the samples at low concentration resulted in formation of the daughter ion fragments at m/z 683, 551, and 389. The fragmentation pattern suggested the presence of salicylated ($\Delta m/z$ 803 − 683 = 120) and apiosylated ($\Delta m/z$ 683 − 551 = 132) hexose units ($\Delta m/z$ 551 − 389 = 162). The position of the salicyl residue on the second sugar unit (apiosyl) was indicated by the absence of the ion at m/z 671 of a salicylated hexose ($\Delta m/z$ 671 − 120 = 551), therefore, the possible attachment position of the salicyl substituent would be the C-5″ carbon of the apiosyl unit. The absorbances at λ 537 and 327 nm (1:0.15) confirmed the lack of any hydroxycinnamoyl moiety and the structure of **A7**/**A7′** was tentatively determined as 5″-*O*-salicyl-2′-*O*-apiosyl-betanin/isobetanin. In addition, **A7**/**A7′** coeluted with the same tentatively characterized pigments derived in the recent study on *P. americana* berry extract (*30*). The relatively highest content of **A7**/**A7′** was found in *R. rhombea*.

Table 1. Chromatographic, Spectrophotometric, and Mass Spectrometric Data of the Analyzed Pigments Found in Stressed Cactus Stems

no.	compound	t_R (min)	λ_{max}[a] (nm), I	λ_{max}[b] (nm), II	abs ratio II:I	m/z $[M+H]^+$	m/z from MS/MS of $[M+H]^+$
A1	betanidin 5-*O*-β-glucoside (betanin)	15.2	–	535	–	551	389
A1′	isobetanidin 5-*O*-β-glucoside (isobetanin)	16.3	–	535	–	551	389
A2	2′-*O*-apiosyl-betanin	16.7	–	536	–	683	551; 389
A2′	2′-*O*-apiosyl-isobetanin	17.7	–	536	–	683	551; 389
A3	6′-*O*-malonyl-betanin (phyllocactin)	18.0	–	536	–	637	619; 593; 551; 389
A4	4′-*O*-malonyl-betanin	18.6	–	536	–	637	619; 593; 551; 389
A3′	6′-*O*-malonyl-isobetanin (isophyllocactin)	19.2	–	536	–	637	619; 593; 551; 389
A5	2′-*O*-apiosyl-phyllocactin	19.7	–	536	–	769	683; 551; 389
A6	2′-*O*-Apiosyl-4′-*O*-malonyl-betanin	20.0	–	536	–	769	683; 551; 389
A5′	2′-*O*-apiosyl-isophyllocactin	20.4	–	536	–	769	683; 551; 389
A7	5″-*O*-salicyl-2′-*O*-apiosyl-betanin[c]	21.8	–	542	–	803	683; 551; 389
A7′	5″-*O*-salicyl-2′-*O*-apiosyl-isobetanin[c]	22.5	–	542	–	803	683; 551; 389
A8	5″-*O*-*E*-feruloyl-2′-*O*-apiosyl-betanin	24.9	326	547	1:0.46	859	683; 551; 389
A9	5″-*O*-*E*-sinapoyl-2′-*O*-apiosyl-betanin	25.1	327	548	1:0.53	889	683; 551; 389
A8′	5″-*O*-*E*-feruloyl-2′-*O*-apiosyl-isobetanin	25.4	327	547	1:0.49	859	683; 551; 389
A9′	5″-*O*-*E*-sinapoyl-2′-*O*-apiosyl-isobetanin	25.4	328	548	1:0.48	889	683; 551; 389
A10	5″-*O*-*E*-feruloyl-2′-*O*-apiosyl-phyllocactin[d]	26.7	328[d]	551[d]	1:0.49[d]	945[d]	769; 683; 551; 389
A11	5″-*O*-*E*-sinapoyl-2′-*O*-apiosyl-phyllocactin[d]	26.7	328[d]	551[d]	1:0.49[d]	975[d]	769; 683; 551; 389
A10′	5″-*O*-*E*-feruloyl-2′-*O*-apiosyl-isophyllocactin[c,d]	26.9	331[d]	551[d]	1:0.53[d]	945[d]	769; 683; 551; 389
A11′	5″-*O*-*E*-sinapoyl-2′-*O*-apiosyl-isophyllocactin[c,d]	26.9	331[d]	551[d]	1:0.53[d]	975[d]	769; 683; 551; 389

[a] λ_{max} of betacyanins in the visible range. –, no absorbance band. [b] λ_{max} of hydroxycinnamoyl moiety (HCA/I). –, no absorbance band. [c] Tentatively identified. [d] Due to co-elution of the peak pairs **A10**/**A11** and **A10′**/**A11′**, the spectrophotometric and mass spectrometric data were not unambiguous.

Table 2. Total Contents and Relative Concentrations of Betacyanins in Stressed Cactus Stems

	relative betacyanin concn[a] (%)						
peak[b]	*Hylocereus polyrhizus*	*Selenicereus megalanthus*	*Epiphyllum phyllanthus*	*Rhipsalis regnellii*	*Rhipsalis rhombea*	*Rhipsalis houlentianum*	*Schlumbergera* × *buckleyi*
A1	3.8 ± 0.67	15.4 ± 2.0	2.8 ± 0.51	8.5 ± 1.4	20.2 ± 2.6	28.5 ± 4.7	5.2 ± 0.55
A1′	4.6 ± 0.63	18.4 ± 2.6	1.8 ± 0.33	5.5 ± 0.68	13.9 ± 2.5	12.2 ± 2.1	2.5 ± 0.32
A2	9.5 ± 1.3	18.2 ± 2.6	10.7 ± 2.2	24.7 ± 3.6	5.7 ± 0.67	10.8 ± 2.0	48.0 ± 5.6
A2′	2.3 ± 0.38	16.7 ± 3.0	7.0 ± 1.4	11.3 ± 1.7	3.7 ± 0.57	5.7 ± 1.1	11.7 ± 1.3
A3	1.3 ± 0.25	0.70 ± 0.13	0.86 ± 0.16	4.1 ± 0.76	1.2 ± 0.15	17.0 ± 3.0	2.2 ± 0.32
A4	0.11 ± 0.015	nd	nd	0.41 ± 0.061	–	4.1 ± 0.73	0.12 ± 0.013
A3′	0.95 ± 0.13	1.2 ± 0.18	0.37 ± 0.058	2.5 ± 0.31	1.2 ± 0.23	12.7 ± 2.1	0.95 ± 0.097
A5	1.3 ± 0.18	4.1 ± 0.59	6.1 ± 0.92	11.7 ± 2.1	2.5 ± 0.33	2.0 ± 0.31	8.3 ± 1.2
A6	0.29 ± 0.049	0.05 ± 0.011	0.27 ± 0.044	0.62 ± 0.097	0.07 ± 0.017	0.48 ± 0.15	0.69 ± 0.19
A5′	0.95 ± 0.12	2.5 ± 0.41	4.6 ± 1.0	5.3 ± 0.79	1.6 ± 0.24	2.7 ± 0.55	4.0 ± 0.57
A7	1.5 ± 0.22	0.81 ± 0.093	1.7 ± 0.37	nd	5.7 ± 0.78	nd	0.49 ± 0.063
A7′	1.3 ± 0.22	2.4 ± 0.42	1.1 ± 0.15	nd	5.0 ± 0.94	nd	0.32 ± 0.036
A8	13.3 ± 2.1	4.6 ± 0.78	7.5 ± 1.4	0.56 ± 0.091	9.3 ± 1.3	1.1 ± 0.25	5.8 ± 0.73
A9	8.7 ± 1.7	4.8 ± 0.67	21.6 ± 4.0	6.4 ± 1.0	5.8 ± 0.76	0.72 ± 0.15	1.8 ± 0.24
A8′	22.3 ± 4.4	4.5 ± 0.77	7.3 ± 1.3	0.61 ± 0.074	12.9 ± 1.9	1.0 ± 0.15	4.3 ± 0.49
A9′	16.4 ± 2.6	3.8 ± 0.56	19.0 ± 3.5	8.7 ± 1.3	6.8 ± 1.1	0.63 ± 0.14	2.0 ± 0.24
A10	2.8 ± 0.36	0.54 ± 0.17	2.6 ± 0.52	3.0 ± 0.53	1.7 ± 0.31	0.23 ± 0.053	0.85 ± 0.13
A11	2.0 ± 0.28	0.31 ± 0.055	1.3 ± 0.18	2.0 ± 0.22	0.72 ± 0.11	nd	0.14 ± 0.018
A10′	4.5 ± 0.61	0.81 ± 0.13	2.3 ± 0.51	2.5 ± 0.49	1.5 ± 0.26	0.14 ± 0.034	0.47 ± 0.046
A11′	2.1 ± 0.47	0.18 ± 0.027	1.1 ± 0.21	1.6 ± 0.26	0.51 ± 0.09	nd	0.17 ± 0.021
total concn[c] (μg/g)	11.7 ± 0.87	39.2 ± 2.8	3.9 ± 0.52	23.3 ± 1.4	17.5 ± 1.6	6.1 ± 1.1	52.4 ± 3.7

[a] Relative concentrations are expressed as percentage of total peak area (average of three measurements). nd, not detected. [b] For all peak assignments, see **Table 1**. [c] Total concentration of betacyanins in betanin equivalents.

Another highly abundant betacyanin **A2** showed a protonated molecular ion at m/z 683 and its daughter ion fragments at m/z at 551 and 389 using positive ion mode LC-MS/MS. The mass difference between **A2** (m/z 683) and betanin **A1** (m/z 551) suggested the presence of an additional pentose moiety (apiosyl). In addition, from the ratio of the absorbances at 539 and 327 nm (1:0.16) the presence of hydroxycinnamoyl residues as acylating moieties in **A2** was excluded (*11*) and no other organic acyl residue was identified. Cochromatography of **A2** with the standards obtained from *Hylocereus* species (*11*) and *P. americana* berries (*30*) confirmed the identity of the pigment as 2′-*O*-apiosyl-betanin. The highest concentration of **A2**/**A2′** was found in stressed *Schl.* × *buckleyi*.

Compounds **A2**/**A2′**–**A8**/**A8′**–**A9**/**A9′** presented the most characteristic pattern of betacyanins in the stressed tissues, forming in some cases the most abundant group of pigments, which was noted for the first time in the cactus samples. Interestingly, the previous study confirmed that **A8**/**A8′** were the dominating pigments in stems of *P. americana* as well as in cell cultures derived from explants of the stems (*29*). In addition, our recent study on *P. americana* betacyanins confirmed that **A8**/**A8′** was accompanied by **A9**/**A9′** (*31*). This fact in connection with the

Table 3. Chromatographic, Spectrophotometric, and Mass Spectrometric Data of Betacyanins in the Hibotan Scions

no.	compound	t_R (min)	λ_{max}[a] (nm), I	λ_{max}[b] (nm), II	abs ratio II:I	m/z $[M + H]^+$	m/z from MS/MS of $[M + H]^+$
B1	(glucosyl)-(glucosyl)-betanin[c]	12.6	–	534	–	875	713; 551; 389
B2	betanidin 5-*O*-β-sophoroside	12.8	–	536	–	713	551; 389
B3	(rhamnosyl)-(apiosyl)-betanin[c,d]	13.0	–	~536[d]	–	829[d]	–
B1′	(glucosyl)-(glucosyl)-isobetanin[c]	13.3	–	534	–	875	713; 551; 389
B2′	isobetanidin 5-*O*-β-sophoroside	13.6	–	536	–	713	551; 389
a	17-decarboxylated **B2**	13.7	–	~505	–	669	507; 345
B3′	(rhamnosyl)-(apiosyl)-isobetanin[c,d]	13.8	–	~536[d]	–	829[d]	–
B4	betanidin 5-*O*-β-glucoside (betanin)	14.5	–	535	–	551	389
a′	17-decarboxylated **B2′**	14.9	–	505	–	669	507; 345
B5	betanidin 6′-*O*-malonyl-5-*O*-β-sophoroside	15.2	–	536	–	799	755; 713; 637; 551; 389
B4′	isobetanidin 5-*O*-β-glucoside (isobetanin)	15.7	–	535	–	551	389
B5′	isobetanidin 6′-*O*-malonyl-5-*O*-β-sophoroside	15.9	–	536	–	799	755; 713; 637; 551; 389
B6	2′-*O*-apiosyl-betanin	16.0	–	536	–	683	551; 389
B7	betanidin 4′-*O*-malonyl-5-*O*-β-sophoroside	16.2	–	536	–	799	755; 713; 637; 551; 389
b/b′	2-decarboxylated **B2/B2′**	16.3	–	532	–	669	507; 345
B6′	2′-*O*-apiosyl-isobetanin	16.9	–	536	–	683	551; 389
B7′	isobetanidin 4′-*O*-malonyl-5-*O*-β-sophoroside	17.0	–	536	–	799	755; 713; 637; 551; 389
B8	phyllocactin	17.3	–	536	–	637	619; 593; 551; 389
B9	betanidin 6-*O*-β-glucoside (gomphrenin I)	17.4	–	538	–	551	389
B10	4′-*O*-malonyl-betanin	17.9	–	536	–	637	619; 593; 551; 389
B8′	isophyllocactin	18.4	–	536	–	637	619; 593; 551; 389
B9′	isobetanidin 6-*O*-β-glucoside (Isogomphrenin I)	18.6	–	538	–	551	389
B11	2′-*O*-apiosyl-phyllocactin	18.9	–	536	–	769	683; 551; 389
B10′	4′-*O*-malonyl-isobetanin	19.3	–	536	-	637	619; 593; 551; 389
B11′	2′-*O*-apiosyl-isophyllocactin	19.8	–	536	–	769	683; 551; 389
B12	5″-*O*-salicyl-2′-*O*-glucosyl-betanin[c]	21.4	–	542	–	833	713; 671; 551; 389
B12′	5″-*O*-salicyl-2′-*O*-glucosyl-isobetanin[c]	22.1	–	542	–	833	713; 671; 551; 389
B13	5″-*O*-*E*-feruloyl-2′-*O*-apiosyl-betanin	24.3	326	547	1:0.49	859	683; 551; 389
B14	5″-*O*-*E*-sinapoyl-2′-*O*-apiosyl-betanin	24.5	327	548	1:0.53	889	683; 551; 389
B13′	5″-*O*-*E*-feruloyl-2′-*O*-apiosyl-isobetanin	24.8	327	547	1:0.50	859	683; 551; 389
B14′	5″-*O*-*E*-sinapoyl-2′-*O*-apiosyl-isobetanin	24.8	328	548	1:0.50	889	683; 551; 389
B15	6′-*O*-*E*-feruloyl-2′-*O*-glucosyl-betanin[c]	25.9	325	546	1:0.47	889	727; 551; 389
B16	6′-*O*-*E*-sinapoyl-2′-*O*-glucosyl-betanin[c]	26.3	327	547	1:0.52	919	757; 551; 389
B15′	6′-*O*-*E*-feruloyl-2′-*O*-glucosyl-isobetanin[c]	26.3	325	546	1:0.51	889	727; 551; 389
B16′	6′-*O*-*E*-sinapoyl-2′-*O*-glucosyl-isobetanin[c]	27.0	327	547	1:0.47	919	757; 551; 389

[a] λ_{max} of betacyanins in the visible range. –, no absorbance band. [b] λ_{max} of hydroxycinnamoyl moiety (HCA/I). –, no absorbance band. [c] Tentatively identified. [d] Due to coelution of the peak pairs **B2**/**B3** and **B2′**/**B3′**, the spectrophotometric and mass spectrometric data of **B3**/**B3′** were not unambiguous.

presence of **A8**/**A8′**−**A9**/**A9′** in the peel of fruits of *Hylocereus* species in contrast to the fruit flesh (*11*) suggested that these pigments are the most indicative of the light stress induced in the plant parts exposed to the sunlight.

The chromatographic patterns were completed by other betacyanins. Betanin **A1**/**A1′** (the best known betacyanin) was readily identified by its protonated molecular ion at m/z 551 and coelution with already obtained standards (e.g., from roots of *B. vulgaris* or fruits of *Hylocereus* species) (*1*, *9*). Interestingly, this dominating pigment in many cacti or other plant species was present at relatively low abundance in the stems of *H. polyrhizus*, *E. phyllanthus*, and *Schl.* × *buckleyi* (**Table 2**; **Figure 2**).

The presence of other betacyanins, which were frequently found in cactus fruits or flowers, was noted (*8*−*11*), namely, 6′-*O*-malonyl-betanin (phyllocactin) **A3**/**A3′** and 4′-*O*-malonyl-betanin (acyl migration product of phyllocactin (*31*)) **A4** as well as other apiosylated and malonylated derivatives (2′-apiosyl-phyllocactin **A5**/**A5′**, 2′-*O*-apiosyl-4′-*O*-malonyl-betanin **A6**, and 5″-*O*-*E*-feruloyl-2′-*O*-apiosyl-phyllocactin **A10**/**A10′**), on the basis of their LC-DAD-MS/MS data (**Table 1**) and cochromatography with *Hylocereus* species standards. Detailed inspection of mass chromatograms revealed an analogue of **A10**/**A10′**, which was tentatively identified as 5″-*O*-*E*-sinapoyl-2′-*O*-apiosyl-phyllocactin **A11**/**A11′** on the basis of its protonated molecular ion ($[M + H]^+$) at m/z 975 and the same fragmentation as of **A10**/**A10′**. The coelution of pairs of **A10**/**A10′** and **A11**/**A11′** suggested their similar acylation pattern as in the cases of **A8**/**A8′** and **A9**/**A9′**; however, it prevented unambiguous spectrometric analysis. In contrast to the fruits of *Hylocereus* species, hylocerenin (6′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-betanin), its typical pigment, was not present in any analyzed stressed cactus samples (*9*, *11*).

The results indicate that the already known betacyanins were present in the light-stressed cactus stems; however, in contrast to the fruits or flowers, the fraction of the apiosylated betacyanins **A2**/**A2′**−**A8**/**A8′**−**A9**/**A9′** was elevated and in some cases highly dominating. The latter pigment group (especially **A8**/**A8′**−**A9**/**A9′**) might be an indicator of the stress process in the cactus stems.

Betacyanins in Hibotan Samples. A broad range of color palette in various grafted cacti has been frequently observed (*23*); however, no information about the presence of specific betaxanthins or betacyanins was given. Interestingly, the yellow-orange color was not a result of the formation of betaxanthins, which are usually present in plants together with betacyanins, but was determined by the synthesis of carotenoids. No betaxanthins were detected in any of the analyzed samples. In addition, the violet scions contained some chlorophyll mixed with betacyanins (**Table 3**) and carotenoids, which resulted in a dark appearance of the epiderm in contrast to the pink scions of bright appearance and the same total level of betacyanins, which contained possibly low levels of carotenoids and no chlorophyll.

The total concentrations of betacyanins in the whole analyzed scions were in the range of 2.4−17.4 μg/g (**Table 4**); however, their

Table 4. Total Contents and Relative Concentrations of Betacyanins in Three Hibotan Scions

peak[b]	compound	relative betacyanin concn[a] (%)		
		violet Hibotan	pink Hibotan	red Hibotan
B1	(glucosyl)-(glucosyl)-betanin	nd	0.19 ± 0.029	0.42 ± 0.078
B2	betanidin 5-*O*-β-sophoroside	43.1 ± 8.7	23.7 ± 4.7	34.4 ± 5.4
B3	(rhamnosyl)-(apiosyl)-betanin	nd	nd	0.16 ± 0.019
B1′	(glucosyl)-(glucosyl)-isobetanin	nd	0.14 ± 0.023	0.23 ± 0.027
B2′	isobetanidin 5-*O*-β-sophoroside	40.8 ± 9.2	16.8 ± 2.3	22.6 ± 4.1
B3′	(rhamnosyl)-(apiosyl)-isobetanin	nd	nd	0.13 ± 0.022
B4	betanidin 5-*O*-β-glucoside (betanin)	2.9 ± 0.62	8.8 ± 1.3	10.5 ± 1.6
B5	betanidin 6′-*O*-malonyl-5-*O*-β-sophoroside	2.6 ± 0.66	0.69 ± 0.12	2.7 ± 0.37
B4′	isobetanidin 5-*O*-β-glucoside (isobetanin)	1.3 ± 0.26	9.3 ± 1.7	7.2 ± 0.81
B5′	isobetanidin 6′-*O*-malonyl-5-*O*-β-sophoroside	3.4 ± 0.63	0.92 ± 0.17	2.3 ± 0.39
B6	2′-*O*-apiosyl-betanin	1.4 ± 0.31	0.89 ± 0.14	0.54 ± 0.094
B7	betanidin 4′-*O*-malonyl-5-*O*-β-sophoroside	nd	nd	0.19 ± 0.027
B6′	2′-*O*-apiosyl-isobetanin	1.1 ± 0.24	0.49 ± 0.11	0.41 ± 0.064
B7′	isobetanidin 4′-*O*-malonyl-5-*O*-β-sophoroside	nd	nd	0.17 ± 0.02
B8	phyllocactin	nd	1.1 ± 0.16	1.3 ± 0.17
B9	gomphrenin I	nd	0.57 ± 0.11	1.2 ± 0.13
B10	4′-*O*-malonyl-betanin	nd	0.24 ± 0.038	0.26 ± 0.035
B8′	isophyllocactin	nd	0.55 ± 0.11	1.9 ± 0.28
B9′	isogomphrenin I	nd	0.61 ± 0.15	1.4 ± 0.24
B11	2′-*O*-apiosyl-phyllocactin	nd	0.28 ± 0.051	0.11 ± 0.014
B10′	4′-*O*-malonyl-isobetanin	nd	0.13 ± 0.019	0.22 ± 0.031
B11′	2′-*O*-apiosyl-isophyllocactin	nd	0.22 ± 0.034	0.11 ± 0.017
B12	5′′-*O*-salicyl-2′-*O*-apiosyl-betanin	nd	0.33 ± 0.059	0.078 ± 0.014
B12′	5′′-*O*-salicyl-2′-*O*-apiosyl-isobetanin	nd	0.25 ± 0.043	0.066 ± 0.0092
B13	5′′-*O*-*E*-feruloyl-2′-*O*-apiosyl-betanin	0.96 ± 0.22	1.6 ± 0.34	0.10 ± 0.014
B14	5′′-*O*-*E*-sinapoyl-2′-*O*-apiosyl-betanin	0.85 ± 0.23	4.5 ± 0.85	0.44 ± 0.058
B13′	5′′-*O*-*E*-feruloyl-2′-*O*-apiosyl-isobetanin	0.48 ± 0.12	2.2 ± 0.48	0.07 ± 0.012
B14′	5′′-*O*-*E*-sinapoyl-2′-*O*-apiosyl-isobetanin	0.39 ± 0.094	5.0 ± 1.0	0.22 ± 0.025
B15	6′-*O*-*E*-feruloyl-2′-*O*-glucosyl-betanin	0.24 ± 0.041	5.5 ± 1.1	3.3 ± 0.37
B16	6′-*O*-*E*-sinapoyl-2′-*O*-glucosyl-betanin	0.22 ± 0.044	4.5 ± 0.92	2.4 ± 0.45
B15′	6′-*O*-*E*-feruloyl-2′-*O*-glucosyl-isobetanin	0.19 ± 0.045	6.0 ± 1.1	3.0 ± 0.38
B16′	6′-*O*-*E*-sinapoyl-2′-*O*-glucosyl-isobetanin	0.17 ± 0.04	4.4 ± 0.87	2.0 ± 0.33
total concn[c] (μg/g)		2.4 ± 0.47	3.4 ± 0.53	17.4 ± 2.9

[a] Relative concentrations are expressed as percentage of total peak area (average of three measurements). nd, not detected. [b] For all peak assignments, see **Table 3**. [c] In betanin equivalents.

concentrations in the epidermal layers should be much higher. In the Hibotan samples betanin was also not the prevailing betacyanin as in the stressed samples (**Figure 3**), but, in contrast to the stressed samples, another polar pigment dominated (**B2**/**B2′**). Due to the differences of lipophilic properties between betacyanins and carotenoids, their separation was accomplished during the purification step on the C18 chromatographic column.

The chromatograms in **Figure 3** depict typical betacyanin profiles in the Hibotan samples, which were purified and concentrated. Some of the betacyanins detected previously in the stressed samples were also present in the grafted cacti, in some cases indicating a possible similarity of reaction to a light stress. These pigments were betanin **B4**/**B4′**, 2′-*O*-apiosyl-betanin **B6**/**B6′**, phyllocactin **B8**/**B8′**, 4′-*O*-malonyl-betanin **B10**/**B10′**, and 2′-*O*-apiosyl-phyllocactin **B11**/**B11′** as well as the most indicative of light stress, 5′′-*O*-*E*-feruloyl-2′-*O*-apiosyl-betanin **B13**/**B13′** and 5′′-*O*-*E*-sinapoyl-2′-*O*-apiosyl-betanin **B14**/**B14′**. The identity of the pigments was confirmed by their characteristic spectrophotometric and spectrometric properties (**Table 3**) and coelution experiments with the authentic standards as in the study on the stressed cacti. Interestingly, no traces of 5′′-*O*-salicyl-2′-*O*-apiosyl-betanin **A7**/**A7′** (which was present in the stressed samples (**Tables 1** and **2**)) were detected in the Hibotan epiderm. However, it was possible that prolonged exposure of the grafted cacti to sunlight would enable the biosynthesis of **A7**/**A7′** or increase the content of the other stress-indicative pigments (**A2**/**A2′**−**A8**/A8′−**A9**/**A9′** from the stress study). This was not confirmed during a 2 month exposure study; however, it cannot be excluded that the period of exposure was too short, so further experiments on the stressed Hibotan cacti should be performed.

Interestingly, another salicylated betacyanin, **B12**/**B12′**, was tentatively identified as 2′-*O*-glucosyl-6′-*O*-salicyl-betanin/isobetanin in the red and pink Hibotan epiderm. The MS/MS fragmentation pattern of **B12**/**B12′** suggested the presence of a salicylated ($\Delta m/z$ 833 − 713 = 120) dihexose unit ($\Delta m/z$ 713 − 389 = 2 × 162). The position of the salicyl residue on the first hexose unit was suggested by a loss of one hexose ($\Delta m/z$ 833 − 671 = 162) and a further cleavage of the salicyl moiety ($\Delta m/z$ 671 − 551 = 120). The presence of a hydroxycinnamoyl residue was also excluded on the basis of the ratio of the absorbances at λ 537 and 327 nm (1:0.15). During the coelution experiments **B12**/**B12′** was identified as the same tentatively characterized pigments derived in the recent study on *P. americana* berry extract (*30*); therefore, pigments **A7**/**A7′** from the stress study as well as **B12**/**B12′** are possibly salicylated betacyanins found in few pigment sources.

The grafted cactus epiderm contained also other pigments that were not detected in the stressed samples. A group of recently analyzed polar pigments in *Mammillaria* fruits (*12*) and flowers (data not shown) was identified in the epiderm, namely, betanidin 5-*O*-β-sophoroside **B2**/**B2′**, betanidin 6′-*O*-malonyl-5-*O*-β-sophoroside (mammillarinin) **B5**/**B5′**, and betanidin 4′-*O*-malonyl-5-*O*-β-sophoroside (a tentatively identified acyl migration product of mammillarinin) **B7**/**B7′**, on the basis of their

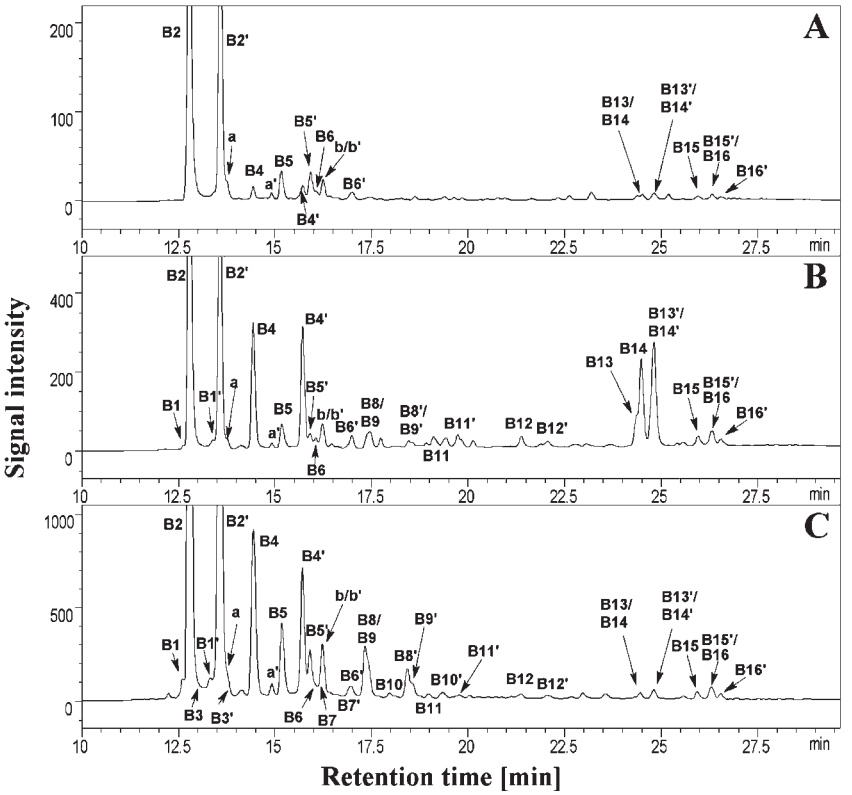

Figure 3. HPLC elution profiles of betacyanins (λ = 538 nm) in Hibotan differently colored scions: (**A**) violet; (**B**) pink; (**C**) red.

analytical data (**Table 3**) and the coelution with *Mammillaria* fruit pigments.

Recent investigations revealed the phenomenon of acyl migration in betacyanins (*31*) resulting in the formation of a series of 4′-*O*- and 3′-*O*-acylated stereoisomers. In the pigmented Hibotan samples, the 4′-*O*-malonylated derivatives of mammillarinin (**B7**/**B7′**) and phyllocactin (**B10**/**B10′**) were detected. These compounds were characterized by the same $[M + H]^+$ m/z and λ_{max} values but were eluted later than the starting 6′-*O*-acylated forms (**Figure 3**).

A polar betacyanin **B3**/**B3′** characterized by the $[M + H]^+$ m/z value of 829 was eluted as a chromatographic peak shoulder of **B2**/**B2′**, respectively, and its low abundance prevented further structure elucidation. However, its very low retention time suggested the presence of unacylated betacyanin derivative, and its high $[M + H]^+$ m/z value indicated three glycoside moieties in the pigment structure; therefore, the tentatively assigned structure might comprise a pentosyl (most probably the apiosyl − 132) and deoxy-hexosyl (e.g., rhamnosyl − 146) attached to the betanin unit ($\Delta m/z$ 829 − 146 = 683; 683 − 132 = 551). Thus, the compound **B3**/**B3′** can be possibly assigned as (rhamnosyl)-(apiosyl)-betanin. The presense of rhamnosyl derivatives of betacyanins had been considered in the previous studies, but no further stucture elucidation was performed (*32*, *33*).

The pigment **B9**/**B9′,** isomeric to betanin **B4**/**B4′**, was identified as betanidin 6-*O*-β-glucoside (gomphrenin I), which was the only 6-*O*-glucosylated derivative of betanidin found in the Hibotan samples. The slight bathochromic shift in the case of **B9**/**B9′** suggested the 6-*O*-glucosylation position, and its identification was completed after the cochromatography with the standard derived from *Gomphrena globosa* flowers (*34*).

In addition to the polar betacyanins, new much more lipophilic pigments were identified in the chromatograms and assigned as 6′-*O*-*E*-feruloyl-2′-*O*-glucosyl-betanin **B15**/**B15′** and 6′-*O*-*E*-sinapoyl-2′-*O*-glucosyl-betanin **B16**/**B16′**. Their λ_{max} 546−547 nm and the second absorption maximum at $\lambda_{max\text{-}HCA}$ 325−327 nm with their absorbance ratio of ca. 0.5 suggested the presence of one hydroxycinnamoyl moiety in each molecule. The $[M + H]^+$ m/z values were 889 and 919, respectively, indicating the presence of glucosylated ($\Delta m/z$ 889 − 727 = 162) and feruloylated ($\Delta m/z$ 727 − 551 = 176) betanin **B15**/**B15′** as well as glucosylated ($\Delta m/z$ 919 − 757 = 162) and sinapoylated ($\Delta m/z$ 757 − 551 = 206) betanin **B16**/**B16′**. The presence of **B15**/**B15′**, which was not detected in cactus species, was supported by its coelution with a compound derived from a salt-tolerant halophyte, *M. crystallinum* L. (*21*, *22*). This pigment had been detected in light-induced stress experiments in *M. crystallinum* L. together with other feruloylated betacyanins (*21*, *22*).

Further comparison of the betacyanin profiles in the nonstressed and stressed cacti by light and other factors will help in understanding the formation routes of the pigments.

This paper characterized for the first time betacyanins, which, together with carotenoids, determine the color of the Hibotan cactus scions. The interesting pattern of the polar betacyanins was completed by the more lipophilic betacyanins acylated with ferulic and sinapic acid in the Hibotan and stressed cacti. The presence of some feruloylated and sinapoylated pigments may be indicative of light stress. Further research on betacyanins in the nonstressed and stressed cacti by light and other factors will help in understanding the formation routes of the pigments.

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