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Genotypic variation of volatile compounds from flowers of gentians

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Gentians (*Gentiana triflora*, *G. scabra*) are one of the most important ornamental plants, however, repellent odor emitted from their flowers makes them undesirable in indoor floral utilization. It is necessary to understand the component of their flower scent for breeding of new cultivars without repellent odor. Floral scent compounds of gentians were analyzed by the method of Head Space-Solid Phase Micro Extraction with GC-MS (HS-SPME/GC-MS). The level of scent emission from gentian flowers increased with the age of the flower and reached a maximum at three days after anthesis and decreased thereafter. Their flowers emitted volatiles throughout the day, and the amounts were higher at night than during the day. A total of 98 compounds were detected in 13 genotypes examined, and quantitative and qualitative variations were found. Of these compounds, several kinds of lilac aldehydes (terpenoids) were only detected in *G. scabra*. The results of principal component analysis showed that cultivars/lines classified in the same species were grouped with each other. Of the 13 genotypes, Ashiro-no-Natsu emitted the most abundant volatiles and has strong unpleasant odor. 2-Methylbutanoic acid is considered to be one of the major constituents responsible for the unpleasant odor of gentians.

Key Words: Gentian, *Gentiana triflira*, *G. scabra*, floral scent, volatile compounds, HS-SPME/GC-MS.

Introduction

Gentiana, a genus belonging to the family Gentianaceae, consists of about 400 species. They are distributed mainly in mountain area of temperate regions of Asia, Europe, and the Americas. Gentians come into bloom from early summer to late autumn, and several species of *Gentiana* are used as economically important flowers and ornamental plants (Kohlein 1991).

Practical cultivation of gentians (*G. triflora* and *G. scabra*) as an ornamental flower in Japan started in the 1950s (Yoshiike 1992). In Japan, since breeding of the cultivar 'Iwate' in 1977, which is the F1 hybrid between *G. triflora* and *G. scabra*, many cultivars of gentians as cut flower and pot flower have been bred by intra- and/or interspecific hybridization mainly using two species, *G. triflora* and *G. scabra* (Takahata *et al.* 1995, Nishihara *et al.* 2008). Some traits such as flower color, shape and cropping season have been improved as main breeding objectives. On the other hand, gentian flowers have repellent odor emitted from the inflorescences, which make them undesirable as indoor floral utilization. In order to expand the demand for gentian flowers, the improvement of cultivars without unpleasant odor and/or with pleasant scent is important. However, few investigations of volatile compounds emitted by the flower of gentian have been reported yet, except for one report by Georgieva *et al.* (2005) who detected volatiles from flowers and leaves of three other *Gentiana* spp. (*G. lutea*, *G. punctata* and *G. asclepiadea*).

Floral scent is a composite character that is determined by a complex mixture of low-molecular weight volatile molecules (Knudsen *et al.* 1993). The volatile compounds produced by plants are responsible for multiple interactions between plants and other organisms (Vainstein *et al.* 2001). Floral scent is an important signal for chemical communication between flowering plants and pollinators (Pellmyr and Thien 1986). Therefore, floral scent is an important trait for the production of fruits and seeds in agricultural and horticultural crops. Scented flowers also constitute a commodity with aesthetic and emotional value and hence it is interesting to improve or modify the flower scent, although flower scent has hardly been a target trait in breeding programs. It is reported that flower scent is lacking in most modern varieties (Vainstein *et al.* 2001). Hence the regulation of floral scent is an agriculturally and horticulturally important subject.

In this study, we characterize the flower scent of gentian using floral headspace collection method coupled with gas chromatography-mass spectrometry (GC-MS). In order to optimize analytical conditions of floral scent of gentian, we first selected the SPME (Solid-Phase Micro Extraction) fiber, which most effectively extracts volatile compounds from flowers. Then we determined the difference of flower scent released from different floral developmental stages and the diurnal change of emission. We also elucidated the genotypic variation of flower scent of gentians, which confer useful information for the breeding of gentians.

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Material and Methods

Plant materials

Thirteen cultivars/lines of gentians, consisting of six cultivars/lines of *G. triflora*, four of *G. scabra*, one cultivar of *G. triflora* × *G. pneumonante*, one cultivar of *G. scabra* × *G. pneumonante*, and one cultivar of (*G. triflora* × unknown) × unknown, were used in this study (Table 1). They were grown at the experimental field of Hachimantai City Floricultural Research and Development Center in Japan.

HS-SPME

The volatile compound constituents were analyzed by Headspace Solid-Phase Micro Extraction (HS-SPME) coupled to GC-MS. The HS-SPME procedures were performed with Supelco SPME fibers, coated with 100 μm polydimethylsiloxane (PDMS) fiber or with 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber. Three flowers were randomly picked from the inflorescence and immediately placed in a 50 ml vial tightly closed with an aluminum cap with a silicone-Teflon rubber septum. After the headspace of the vial reached equilibration at 30°C for 60 min, the fiber was exposed to headspace at 30°C for 60 min. Each experiment had three independent replicates. SPME fiber blanks were always tested between sample analyses.

Optimization of volatile sampling period after anthesis

To determine the amount of volatile compounds emitted at different stages of flower development, flowers of Ashiro-no-Natsu were collected at four different stages of flower development from day 1, 2, 3 and day 5 after anthesis (Fig. 2A), stage 1, one day after flowering; stage 2, two days after flowering; stage 3, three days after flowering; stage 4, five days after flowering. To clarify the temporal variation over a day in emission of volatile compounds, flowers of Ashiro-no-Natsu and Gokuwase collected at 3 h-intervals between 9:00 and 24:00 were analyzed.

GC-MS analysis

The SPME fiber was immediately inserted into the GC injector port, and thermally desorbed at 230°C with a desorption time of 5 min. GC-MS analyses were carried out using a GC-17A (Shimadzu, Japan) gas chromatograph fitted with a polyethylene glycol capillary column (DB-WAX; 60 m in length, 0.25 mm i.d. and 0.25 μm film thickness; J & W Scientific, USA) coupled to a mass spectrometer (QP-2010, Shimadzu, Japan). Helium at a flow rate 0.8 ml/min was used as the carrier gas. Electron ionization mass spectra were obtained at an ionization voltage of 70 eV and a source temperature of 240°C. The oven temperature was programmed from 40°C to 210°C at a rate of 5°C min^{-1}, and then maintained at 210°C for 30 min. The injector was operated in the splitless mode for 0.75 min after injection of the sample.

The MS compound peaks for each material were then integrated using Shimadzu GC-MS solutions software (version 2.21, Shimadzu, Japan). Volatile compounds were tentatively identified using computerized mass spectroscopic libraries (Wiley version 7 database) and the Kovats retention index. In order to determine the Kovats index of the compounds, a mixture of *n*-alkanes (C_6-C_{21}) was used. Further identification was based on comparison of mass spectra and retention times between the compounds detected and authentic compounds analyzed under the same conditions.

Statistical analyses

In order to examine the relationships among the genotypes based on flower scent, we performed principal components analysis (PCA) using either direct or root transformed values of the 98 volatile compounds identified (Table 2). Calculation of PCA was performed with JMP software (Version 8.0, SAS Instituted Inc.).

Results

Determination of optimum conditions on analysis of flower scent

Two different SPME fibers of PDMS and DVB/CAR/PDMS were evaluated to determine which ones more

Table 1. Thirteen cultivaras/lines of gentian used in this study

Genotype	Caltivar/line	Flower color	Blooming seasons
G. triflora	Gokuwase	Blue	June–July
	Ashiro-no-Natsu	Blue	July–August
	Ashiro-no-Aki	Blue	August–September
	Ashiro-no-Hatsuaki	Blue	September
	Ashiro-no-Kaze	Blue	September
	Summer Snow	White	July–August
G.scabra	Ashiro-no-Sawakaze	Blue	September
	GSB	Blue	September
	GSP	Pink	September
	GSW	White	September
(*G. triflora* × unknown) × unknown	Lovely-Ashiro	Pink	July–August
G. triflora × *G. pneumonanthe*	New Hybrid	Blue	July–August
G. scabra × *G. pneumonanthe*	Seisai	Pear blue	August–September

Table 2. Identification of volatile compounds emitted from S3 stage flowers of 13 cultivars/lines of gentians. Values are means of three replications

Compounds	Retention time	RI	Peak area (%) / total peak area												
			Goku-wase	Ashiro-no-Natsu	Ashiro-no-Aki	Ashiro-no-Hatsuaki	Ashiro-no-Kaze	Summer Snow	Lovely-Ashiro	New Hybrid	Seisai	Ashiro-no-Sawakaze	GSB	GSP	GSW
Benzenoids															
Benzene	8.64	841		1.7		1.5				0.8					
Methylbenzene*	11.89	1030	0.6	2.8	0.3	1.1	1.0	6.4	1.3	1.9	0.5	2.8	5.6	5.2	6.1
Ethylbenzene	14.93	1118	0.5	0.9				0.8		0.6		0.6	0.8	0.5	0.7
1,4-Dimethylbenzene	15.81	1145	1.2	1.1	0.7	0.6	0.5	0.8		0.6	0.6	0.4			1.1
1,2-Dimethylbenzene	17.41	1190						0.5		2.0					
Pyridine	17.43	1191	0.5			0.7	0.3	0.9		2.6			0.8	1.3	0.7
Styrene	19.61	1258		1.4	0.4					3.8	0.4				
1,2,4-Trimethylbenzene	20.45	1277	0.5				0.2	1.2			0.5	0.5	1.5	0.7	1.9
Anisole*	22.46	1345	2.9	3.0	0.4	1.6	0.2	1.1		1.1	0.4		0.5		
o-Dichlorobenzene*	25.51	1447			0.8	8.8	0.3			1.1					0.4
m-Dichlorobenzene*	25.55	1450	0.6								0.5		2.0	1.3	2.1
Benzaldehyde	28.06	1537	0.8	2.6	0.7	0.6	0.3	3.1	2.6	0.8	0.6	0.5	0.4		
Methyl benzoate	30.77	1638					1.0	1.1				7.5	14.3	6.2	2.3
1-Phenylethanone*	31.60	1672	1.4	6.9	3.3	0.8	2.2	1.5	1.9	33.8	7.1	0.5	1.5	0.9	
Benzyl acetate*	33.38	1743			0.8	3.2	0.7		3.6				1.7	3.0	5.7
1,4-Dimethoxybenzene*	33.65	1753			1.3	2.4	15.1	1.2	1.7	1.6			0.5		
1-Phenylethanol	35.45	1826		2.2			0.3	1.1		2.6	2.1				
1-Phenylethyl acetate*	36.50	1871		1.7				0.6		0.7					
Benzyl 3-methylbutanoate*	36.69	1879						0.4							
Phenylmethanol*	37.01	1893	0.8	0.5	2.2	2.4	1.3	1.8	2.0	0.6	0.5	0.9	1.3	2.3	1.2
Benzyl pentanoate*	37.41	1910	1.8	1.4	2.3	3.2	0.6	7.2		0.7		0.8	0.8	1.1	0.9
2-Phenylethanol*	37.85	1930		1.1	4.4	1.1	1.6	1.4	1.8	0.6			0.6	1.0	
1,2,3-Trimethoxybenzene*	38.87	1972		0.8	1.0		0.9	1.2							
1-(3-Methoxyphenyl)ethanone *	40.37	2035			0.7		0.4			0.9	0.4	0.8			
			11.6	*28.1*	*19.3*	*28.1*	*26.9*	*32.2*	*15.0*	*56.7*	*13.6*	*15.4*	*32.3*	*23.6*	*23.0*
Fatty acid derivatives															
Hexanal	13.39	1077	1.3	0.5	4.4	1.1	2.2	2.3	5.3	0.9	0.7	0.7	1.3	1.5	0.8
Z-3-Hexenal	15.90	1146					0.4		2.6						
Octan-3-one*	19.51	1257				1.5									
1-Hexyl acetate	20.05	1264			0.4	0.7	0.3			3.6					
Z-3-Hexenyl acetate	21.28	1309		0.2	0.9	0.9	0.3			1.1		1.0	3.5	1.8	1.1
Hexan-1-ol	22.71	1353	3.7	1.6	15.3	4.8	8.7	3.9	4.6	1.2	0.7	1.1	1.4	0.6	0.5
E-Hexan-3-ol*	23.02	1355					0.2								
Z-3-Hexen-1-ol	23.70	1384	2.6	0.7	21.8	4.9	9.6	0.8	7.6	2.1	0.6	1.3	4.4	1.2	0.7
Octan 3-ol*	23.92	1391	0.4	1.2	1.2	11.9	3.7	3.1		0.7			0.6		0.8
Z-5-Octan-3-ol*	24.43	1410			0.6	0.9	4.1	0.4							
1-Octen-3-ol	25.52	1448	0.5	0.3	0.8		5.3	0.8							
Acetic acid	25.65	1451	0.6	0.4				2.0			0.6	3.0			
Hexanoic acid	36.20	1863			0.8						0.6				
			9.0	*4.9*	*46.2*	*26.7*	*34.9*	*13.3*	*20.1*	*9.6*	*3.2*	*7.2*	*11.1*	*5.0*	*4.0*
Terpenoids															
Pinene*	11.21	1009					6.2								
Lilac aldehyde A*	25.78	1452										3.0		2.4	0.9
Lilac aldehyde B*	28.43	1554									5.4	38.3	23.1	20.7	18.8
Lilac aldehyde C*	28.85	1568									0.8	5.2	1.1	6.0	5.5
Lilac aldehyde D*	29.06	1575										4.6	1.3	5.2	3.5
Lilac aldehyde E*	29.77	1599									0.3	4.4	1.8	4.9	5.6
							6.2				*6.5*	*55.5*	*27.4*	*39.2*	*34.4*
Others															
2-Propanone*	5.83	632	2.0	0.3	2.0	3.5	0.8	0.6	24.1	0.4	1.1	1.7	1.6	1.1	1.9
Methyl acetate*	6.01	647	7.3	0.7		1.2				5.2					
2-Butanone*	7.55	463	3.1	0.5	2.5	6.6	0.9	1.0	5.5	4.0	2.0	1.8	2.5	2.2	2.9
2-Methylbutanal*	7.87	786	3.0	1.1	0.5	0.6	0.7	0.8				1.4			0.6
3-Methylbutanal*	7.99	794	0.3	0.3			0.5	0.4				0.6			0.4
Ethanol*	8.65	842	12.5		11.3		5.0	8.4	8.2		0.8	4.5	3.5	6.1	3.7
4-Methyl-1,3-dioxolan-2-one*	9.72	915						1.4	1.7	2.0					
2,3-Butanedione	9.76	919	3.7	3.6	0.4		0.3				9.1	0.9		1.2	1.0
Methyl 3-methylbutanoate*	11.19	1008	0.4					0.5				0.8			
2-Butanol	11.55	1018	0.3		0.5	0.8	0.7		2.4		0.4				

Table 2. (continued)

Compounds	Retention time	RI	Peak area (%) / total peak area												
			Goku-wase	Ashiro-no-Natsu	Ashiro-no-Aki	Ashiro-no-Hatsuaki	Ashiro-no-Kaze	Summer Snow	Lovely-Ashiro	New Hybrid	Seisai	Ashiro-no-Sawakaze	GSB	GSP	GSW
3-Nonene-2-one*	12.43	1047										3.1	0.9	2.6	0.8
Ethyl 3-methylbutaoate	13.12	1066										0.7		0.5	
Undecane*	13.71	1084											0.9	1.5	1.3
2-Methylpropan-1-ol	14.09	1093	0.3	1.4			2.7				2.8				
Pentan-3-ol	14.47	1103			0.5	0.9		0.7	2.2	0.5			1.8		
3-Methylbutyl acetate	14.86	1115					0.3								
2-Methylbutyl acetate*	15.20	1124											0.9	0.5	2.0
Pentan-2-ol	15.38	1132						0.6					1.3	1.1	1.1
1-Butanol	15.91	1147			0.7			0.8		1.2	0.9		2.6	1.2	0.7
2,3-Heptanedione*	15.94	1148	0.7	0.3		1.0									
Pentyl acetate*	16.95	1177					0.2	2.1							
1-Penten-3-ol*	17.00	1178												0.7	1.8
2-Methylpropyl 3-methylbutanoate*	17.47	1192	0.4	0.5											
Dodecane*	17.88	1203						1.6		1.4			0.5	0.9	0.9
2-Methyl-1-butanol	17.92	1204	4.1	7.4	0.8	1.3	1.4	3.1	2.3	1.1			0.5		1.4
3-Methyl-1-butanol	17.97	1204	4.5	5.0	1.0	3.1	2.0			0.5	10.2	1.1			
(1-Methylethyl)benzene	18.37	1219						0.5					0.8	0.4	0.7
3,7-Dimethyl-1-octanol*	18.80	1230								0.6	0.7				
3-Methyl-3-buten-1-ol	19.32	1250			0.7	1.2	0.7	0.3	2.8	0.5	0.7		0.6		0.7
1-Pentanol	19.37	1251		0.3		1.2	1.6	1.0		5.3			0.4	0.7	0.7
Tridecane	19.73	1262											0.5	0.4	0.5
3-Hydroxybutan-2-one	20.66	1291	13.3	28.6	1.9	3.7	1.3	17.3	5.7	7.9	44.0	3.5	0.4	2.6	4.2
Pentyl 3-Methylbutanoate*	20.86	1297	3.0	9.6			0.7	6.0					0.3	0.4	1.4
4-Methyl-1-pentanol*	21.31	1310						0.6	2.0		0.8				
Z-2-Pentenol*	21.50	1316					1.0		2.3				0.3		
Heptan-3-ol*	21.67	1321					0.6								
6-Methyl-5-heptene-2-one*	22.22	1339					0.3								
3-Methyl-3-butenyl-isovalerate*	23.23	1371		0.1				0.4							0.5
1-Tetradecane	23.95	1392											0.3	0.4	
4-Methylhexanol*	25.02	1426			0.2										
Hexyl pentanoate*	25.66	1452													0.3
Heptanol	25.72	1458	3.6	1.3	3.7	7.4	0.4	3.1	3.5	1.2					
6-Methyl-5-hepten-2-ol*	25.93	1465			0.6		0.3								
1-Hexyn-3-ol*	26.40	1479			0.4		1.7								
2-Ethylhexanol*	26.72	1490					2.6				0.8	1.9	1.8	2.0	0.8
Pentadecane	26.89	1496			0.7	0.5	0.2								
Z-3-Hexenyl 2-methylbutyrate*	26.93	1498	0.4												
2,5-Dihydroxybenzaldehyde*	27.09	1503			0.3										
2-Methylpropanoic acid	28.99	1572	3.1	1.3	1.5	1.3		0.5							
3-Methyl-2-butanol*	29.17	1580						1.1							
Butane-2,3-diol	29.18	1581				0.6	0.6				0.5			0.6	
Dimethyl sulfoxide*	29.63	1595						0.4							
2-Methylbutanoic acid	31.71	1680	13.2	4.8	3.9	10.2	3.7			0.8					
4-Ketoisophorone*	32.65	1713			0.5		0.8	1.2	2.2	1.2	0.6		1.1	0.6	
Ethyl 2-mercaptopropionate*	34.88	1801									1.2		5.6	4.7	8.2
			79.4	*67.0*	*34.4*	*45.2*	*32.0*	*54.5*	*64.9*	*33.7*	*76.7*	*21.9*	*29.2*	*32.2*	*38.6*
Total peak area (%)			100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Total peak area			68377	187009	42188	23885	57980	34778	9852	23611	32887	23087	31737	37791	32351

* Tentatively identified

effectively absorbed volatile compounds from flowers of gentian. Comparison of the SPME fibers on absorption efficiencies of seven compounds, 2,3-butanedione, 1,4-dimethylbenzene, *Z*-3-hexenyl acetate, lilac aldehyde A, C, D and E in the flower emission of Ashiro-no-Sawakaze, is shown in Fig. 1A. The DVB/CAR/ PDMS fiber gave higher peak areas in almost all compounds than the PDMS fiber. The total amount of the seven volatile compounds absorbed by the DVB/CAR/PDMS fiber was 4 times higher than that by the PDMS (Fig. 1B), and the number of volatile compounds determined by the DVB/CAR/PDMS fiber was 3.6 times more than that of the PDMS fiber (Fig. 1C). These results indicate that the DVB/CAR/ PDMS fiber is a suitable system for estimating the volatile compounds of gentian flowers. Hence the DVB/CAR/PDMS fiber was selected for further studies.

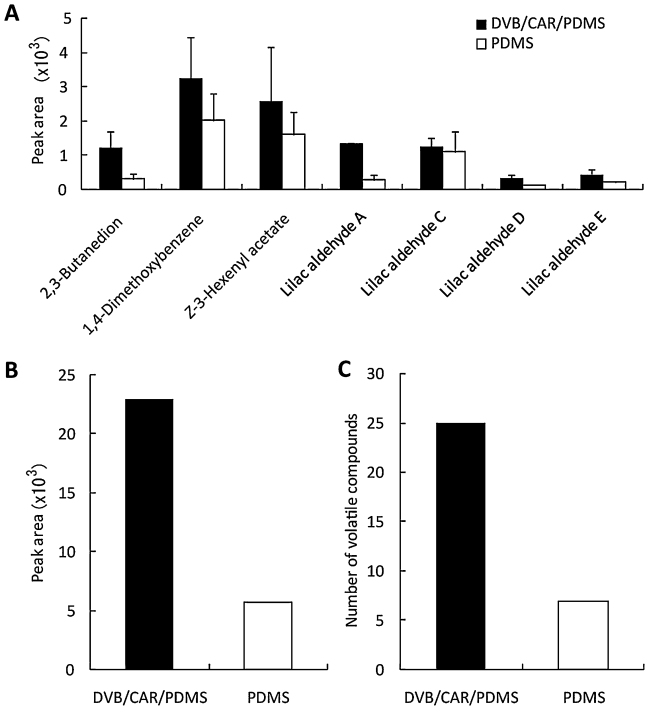

Fig. 1. Effect of SPME fibers on extraction efficiency of volatile compounds emitted from flowers of *G. scabra* cv. Ashiro-no-Sawakaze. (A) The amount of seven compounds. Vertical bars represent SD (n = 3). (B) The total amount of volatile compounds. (C) Number of volatile compounds detected.

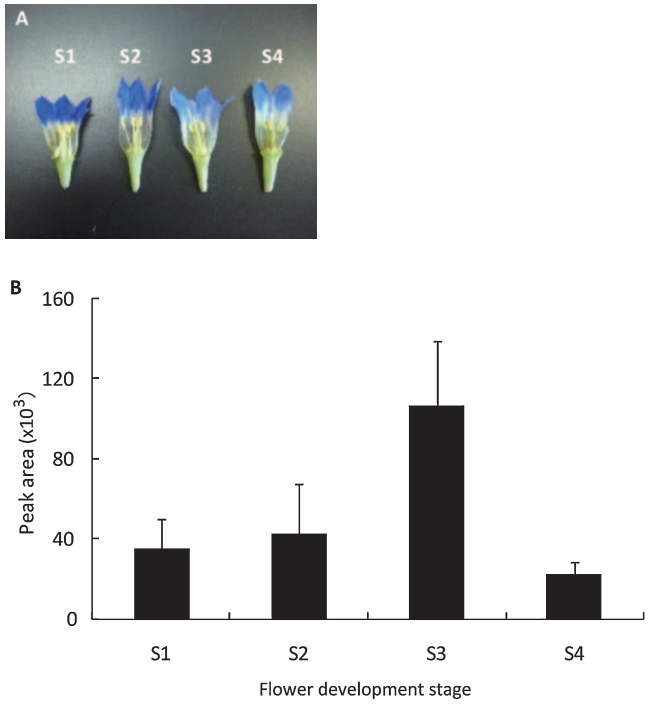

Fig. 2. Changes in the level of floral scent volatiles emitted from flowers of *G. triflora* cv. Ashiro-no-Natsu during the bloom period. (A) Flower developmental stages. Stage 1 (S1), one day after anthesis; Stage 2 (S2), two days after anthesis; Stage 3 (S3), three days after anthesis; Stage 4 (S4), five days after anthesis. (B) Change in the level of volatile compounds during different flower developmental stages. Vertical bars represent SD (n = 3).

Emission of volatile compounds from flowers of different ages from one day to five days after anthesis was examined using Ashiro-no-Natsu. The level of emission increased with the age of flower and reached a maximum three days after anthesis (Fig. 2). The flowers five days after anthesis emitted a small amount of volatiles. To determine the change of volatiles emitted from flowers during a day (9 am to 12 am), volatiles were collected every 3 hours from 9 am to 12 am using flowers of day 3 after anthesis of two cvs. Ashiro-no-Nasu and Gokuwase. Although gentian flowers emitted volatiles throughout a day (Fig. 3), the total amount of volatiles was higher at night than during the day, and reached maximum at 9 pm.

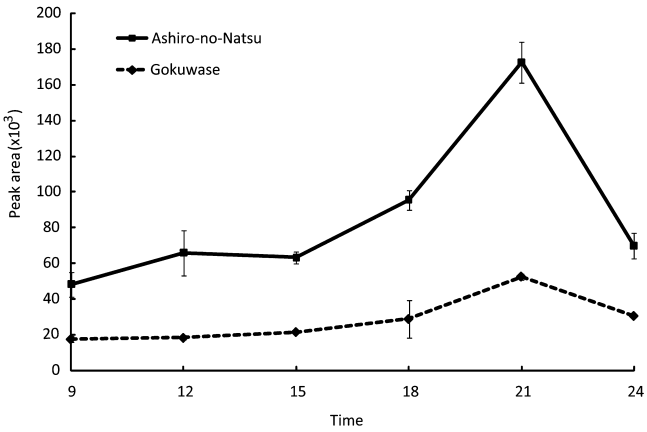

Fig. 3. Time-courses of the emission of volatile compounds from flowers of stage 3 (S3) of *G. triflora* cvs. Ashiro-no-Natsu and Gokuwase. Vertical bars represent SD (n = 3).

Genotypic variation of flower scent profiles

When the volatile compounds of the 13 genotypes were determined by the HS-SPME/GC-MS, the profiles of the volatile compounds showed large genotypic variation. The chemical composition of the gentian floral scents of the 13 cultivars/lines is listed in Table 2. A total of 98 compounds were detected, and 41 of them have been tentatively identified by comparing the mass spectra and retention index with those of Kovats data. The volatiles are classified into three major groups by their biosynthetic origin, that is, benzenoids and phenylpropanoid, fatty acid derivatives, and terpenoids (Knudsen *et al*. 1993). There were qualitative and quantitative variations in the grouped compounds among the 13 genotypes (Fig. 4). Ashiro-no-Natsu emitted the highest amount of volatiles, followed by Gokuwase, whereas Lovely-Ashiro emitted the smallest amounts. The amount of typical compounds in each compound group also varied among genotypes as shown in Fig. 5. Of the three major groups, relatively high amounts of benzenoids were detected in all genotypes of gentian. Terpenoids except pinene were detected only in *G. scabra* and the hybrid cultivar between *G. puneumonate*

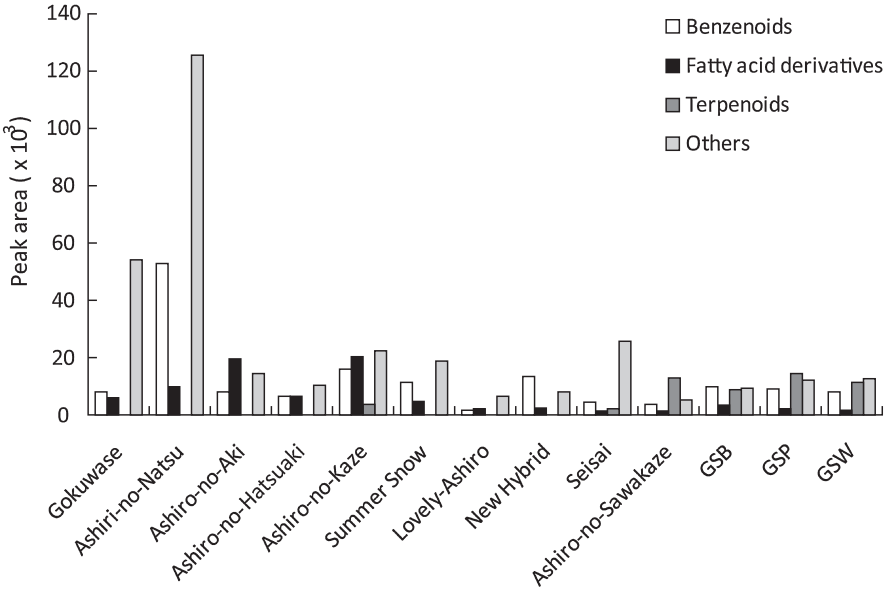

Fig. 4. Genotypic variation in the level of floral scent volatiles on chemical compound groups classified.

and *G. scabra*. Of five kinds of lilac aldehyde in terpenoids, the amount of lilac aldehyde B was highest in all genotypes (Fig. 5). Although fatty acid derivatives were detected in all genotypes, their highest amounts were found in Ashiro-no-Aki and Ashiro-no-Kaze. Of fatty acid derivatives, high levels of *Z*-3-hexen-1-ol and hexan-1-ol were detected in almost all genotypes. Among the other volatile compounds except for the three major groups, 2-methylbutanoic acid, heptanol, 2-methyl-1-butanol and 3-methyl-1-butanol were identified as compounds with high amounts. Ashiro-no-Natsu and Gokuwase emitted higher levels of these four compounds.

Principal component analysis

In order to demonstrate the relationships among the 13 genotypes based on floral scent, principal component analysis was carried out using the value of 98 volatile compounds. Two calculations, using either direct or root-transformed values, gave similar results, and the latter is used for discussion in the present paper. About 45% of total variation was extracted by the first and the second components (Fig. 6). The compounds that were related to unpleasant odor, such as heptanol, 2-methylbutanoic acid and 2-methyl-1-butanol, showed higher plus values of eigenvectors on the first component, while several kinds of lilac aldehyde had higher minus values of eigenvectors. Genotypes having unpleasant odor are distributed on the plus side, and those having lilac aldehyde on the minus side. The highest plus value of the eigenvector on the second component was ethylbenzene, which has an unpleasant odor, and the highest minus one was 1,4-dimethoxybenzene and 1-hexyn-3-ol. As shown in Fig. 6, where the 13 genotypes were projected on the first and second axis, cultivars/lines belonging to the same species are located closely to each other. Cultivars/lines of *G. triflora* are located on the right side. Early varieties such as Gokuwase, Ashiro-no-Natsu and Summer-Snow are on the right upper side, and Ashiro-no-Natsu, in particular, is on most right side. In contrast, all four cultivars/lines of *G. scabra* are located on the second quadrant. Interspecific hybrid cultivars (Lovely-Ashiro, New-hybrid, Seisai) are distributed between *G. triflora* and *G. scabra*.

Discussion

Many plants emit variable floral scents with respect to quality and quantity (Knudsen and Gershenzon 2006, Knudsen *et al.* 2006). These floral scents play many important roles in the interaction between plants and their surroundings; a main one is attraction to pollinators (Dundreva and Pichersky 2000, Vainstein *et al.* 2001). For humans, flowers with delightful scents also have aesthetic values and restorative effects on the mind. In contrast, flowers emitting repellent odor are unpleasant for humans although they are attractive for some pollinators. Gentians are important ornamental flowers, but they are one of the plants emitting repellent odor. Therefore, it is important to breed the cultivars without such repellent odor. In the present study, their volatile compounds were analyzed by HS-SPME/GC-MS method in order to characterize flower scents of gentians.

Our results revealed that the emission of flower scents increased from anthesis to fully open flower, reaching a maximum at three days after anthesis, and decreased at five days anthesis. The flowers four days after anthesis were not analyzed in this study, and the level of emission from this stage flowers remain to be elucidated. Such change has been reported to be a general phenomenon in many species such as *Clarkia breweri*, *Antirrhinum majus* and several roses (Pichersky *et al.* 1994, Dudareva *et al.* 2000, Picone *et al.* 2004, Bergougnoux *et al.* 2007). The emission of scent from opened flower is related to the attraction for pollinators

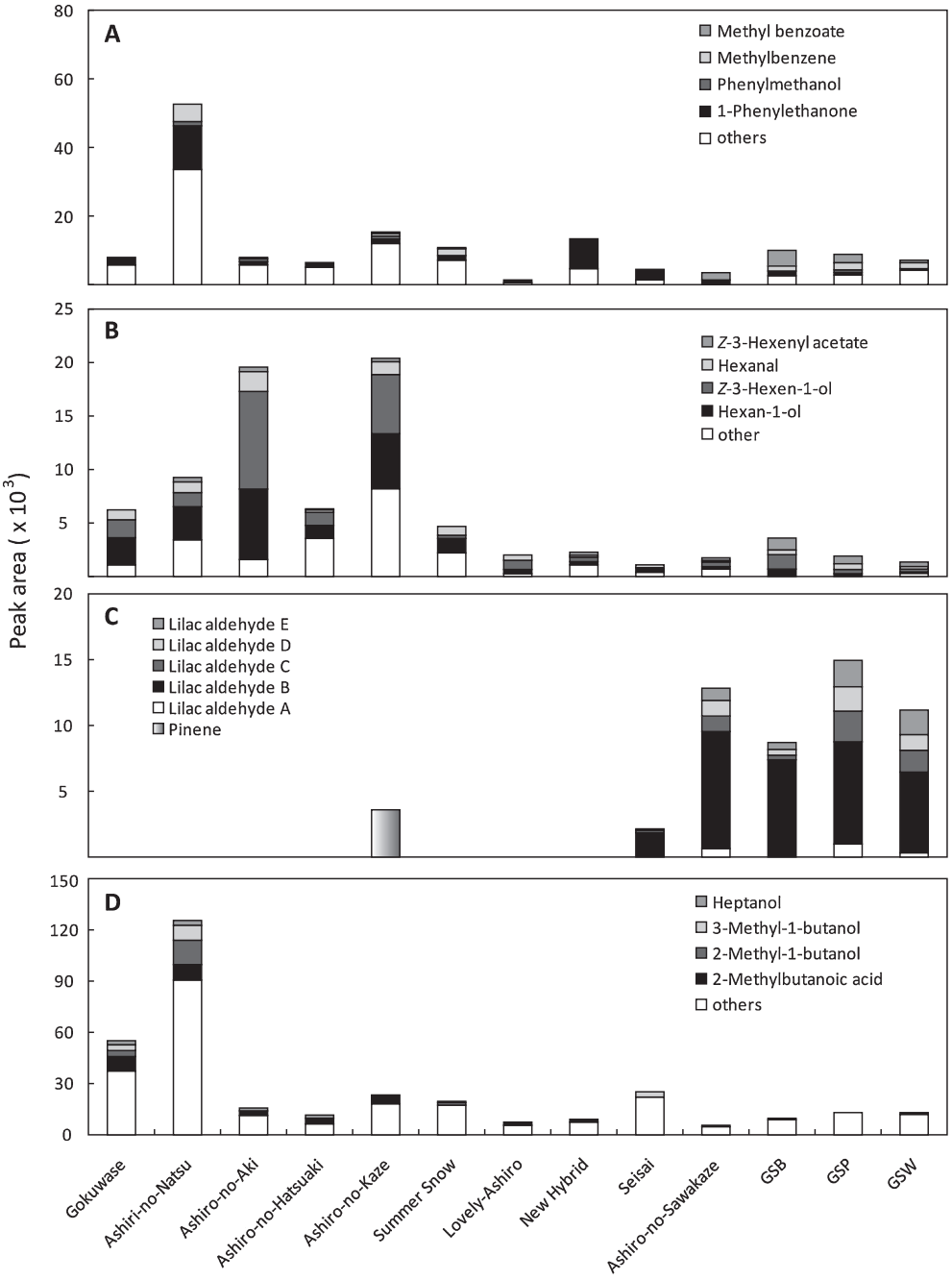

Fig. 5. Genotypic variation in the level of main volatile compounds. (A) Benzenoids, (B) Fatty acid derivatives, (C) Terpenoids, (D) Others.

(Negre *et al.* 2003). Several researchers have reported that the pollination decreased emission of floral scents with the other post-pollination phenomena such as permanent flower closure and color change in several plant species (Tollsten and Bergstrom 1989, Dotterl *et al.* 2005). The reduction of scent emission observed at five days after anthesis in the present study is considered to be attributed to spontaneous reduction by aging or to pollination of flowers. This point remains to be elucidated.

Although gentian flowers emit volatiles during a whole day, the peak time of emission is during the night. Such diurnal oscillations of scent emission have been reported in many plants (Oyama-Okubo *et al.* 2005, Handel-Rhmanim *et al.* 2007). Generally, flowers which pollinate at night tend to have a peak emission during the dark period (Handel-Rhmanim *et al.* 2007). Since gentian is an day-opening flower, it is interesting to clarify the reason why it has such character. Recent studies reported that the diurnal oscillations are regulated by circadian clock or by light directly (Roeder *et al.* 2007, Handel-Rhmanim *et al.* 2007). Further investigations of rhythmic cycles or light regulation in emission of gentian floral scents are needed.

A total of 98 compounds were detected from flowers of gentian by analyzing floral volatiles of 13 genotypes under

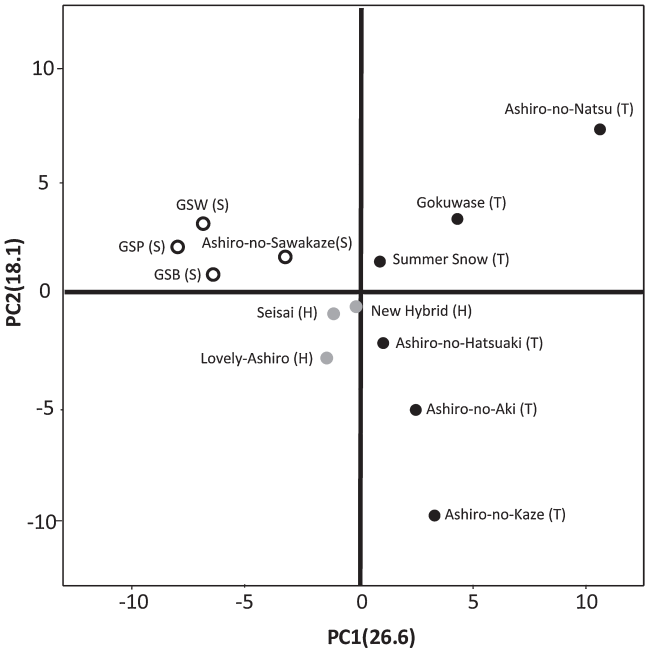

Fig. 6. Scatter diagram of 13 genotypes projected on the first and second axis of principal component analysis based on volatile compounds. The value on parenthesis of each principal component shows proportion. T, *G. triflora*; S, *G. scabra*; H, Hybrid cv.

optimized HS-SPME/GC-MS conditions, and a large genotypic variation was revealed in the compounds. Such variations have been reported in many plants (Andersson *et al.* 2002, Knudsen *et al.* 2006). Although floral scent composition varies qualitatively and quantitatively among genotypes, cultivar/lines classified into the same species have relatively similar traits. This is supported by the results of principal component analysis (PCA). *G. scabra*, which is considered to have the same genome as *G. triflora* because of easy hybridization with *G. triflora*, has unique compounds, terpenoids such as several kinds of lilac aldehyde. A hybrid cultivar using *G. scabra* as a parent also emitted these compounds and the results of PCA showed that the interspecific hybrid cultivar located between their parents. These results suggest that floral scents are inherited traits and lilac aldehydes might be utilized as a marker for discrimination between *G. scabra* and *G. triflora*. These unique compounds in *G. scabra* might be related to the reproductive isolation of this species from sympatric existence of *G. triflora* by different pollinators. Lilac aldehydes, which are not often found in floral scents (Knudsen *et al.* 1993), were reported to be the most critical compound for attraction of moths (Dotterl *et al.* 2006).

Of the 13 genotypes, Ashiro-no-Natsu emitted the most abundant volatiles, and it was located on the most right side in the PCA plot. A preliminary panel test using Ashiro-no-Natsu and New-Hybrid revealed that Ashiro-no-Natsu has strong unpleasant odor (data not shown). The results of PCA showed that the genotypes with large values of the first component had a relatively higher concentration of heptanol, 2-methylbutanoic acid, phenylmethanol, anisole, etc. Of these compounds, 2-methylbutanoic acid is known to be responsible for the compounds of sweaty smell of animals. Sniffing the reference compound of 2-methylbutanoic acid showed that this compound has similar odor to the gentian unpleasant odor. These results indicate that 2-methylbutanoic acid is considered to be one of major constituents responsible for the unpleasant odor of gentian. Methylbutanoic acids are rarely detected in flowers from most species. In gypsophila (*Gypsophila paniculata*), methylbutanoic acids are identified as an unpleasant scent in this species (Nimitkeatkai *et al*. 2005).

As mentioned in Introduction, breeding of gentians has a short history in comparison with that of other ornamental flower plants such as rose, carnation and chrysanthemum. With the progress of flower breeding, flower scent is lacking because of negative correlations between flower scent and target traits such as color, shape and longevity (Vainstein *et al.* 2001). High amounts of flower scents in gentian are probably due to short breeding history of this flower crops. Our study has uncovered qualitative and quantitative variation of floral scent compounds in gentian, and provides important information for gentian breeding. We are currently examining the inheritance of floral scent and variation of floral scent compounds in other species of *Gentiana*.

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