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A Consistent Phenotyping of Floral Scent in *Mitella* (Saxifragaceae) Using Solid-Phase Microextraction

Yudai Okuyama*

Department of Botany, National Museum of Nature and Science,
Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
* E-mail: yokuyama@kahaku.go.jp

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Abstract I established a simple method for quantitatively phenotyping floral scent traits in small-flowered plant species, especially Asian *Mitella* (Saxifragaceae). To test the reliability of the method for comparative studies and examine if the scent traits are under the control of the plant genotype, I used 29 individuals of the F2 generation of the hybrid of *M. furusei* and *M. pauciflora*. The floral scent traits were examined for these plants in the flowering seasons of both 2014 and 2015. Accordingly, I confirmed that the traits phenotyped in the present study were variable among the F2 individuals but stable across the two flowering seasons within each individual. These observations demonstrated that the study system is useful to carry on the genetic analysis of the floral scent traits, which are likely controlling the reproductive isolation in Asian *Mitella*.

Key words: *Heuchera* group, genetics, pollination, quantitative trait, volatile.

Among floral traits that potentially determine the specificity of plant-pollinator interactions, the scent might have been one of the most difficult to measure. Nevertheless, it is crucial to establish a standard method for comparatively phenotype the floral scents among the samples of different plant origins, because only such a method enables the comparative studies on phylogenetics or genetics, which is necessary to clarify how the diversity of the floral scent has been shaped in an evolutionary context.

The *Heuchera* group (Saxifragaceae) is a monophyletic group of small-flowered (mostly 5–10 mm in diameter) perennials that grow in various habitats of North America and East Asia (Soltis *et al.*, 2001). Recently, this plant group has become the focus of the studies on floral diversification driven by plant-pollinator interactions (Okuyama *et al.*, 2008; Thompson *et al.*, 2013). More importantly, floral scents are considered to be the key traits that likely determine the specificity observed therein (Friberg *et al.*, 2013; Okamoto *et al.,* 2015).

Accordingly, here I apply a simple method using solid-phase microextraction (SPME) to quantitatively phenotype the floral scent traits in *Mitella*, which is the genus included in the *Heuchera* group. In this study, I use 29 F2 hybrid *Mitella* plants that are presumably variable in floral scent phenotypes because of different genetic characteristics. By measuring the traits across two flowering years for these plants, I examine if the measure obtained by the method is reproducible enough to allow genetic and/or phylogenetic comparative studies.

Materials and Methods

Plant materials

For the present study, I used the F2 generation of the hybrid of *M. furusei* and *M. pauciflora* that have been prepared for the genetic studies (Okuyama and Akashi, 2013). Twenty nine individuals out of the 222 F2s were randomly chosen

to examine the diversity of floral scent phenotypes and their stability across the two flowering seasons.

Sample preparation and sampling

A series of analytical standard (10, 50, 100, 200, 300, 400, 500, and 1000 μg/μL) was prepared for (RS)-linalool (Wako, Tokyo, Japan) using ethanol as the solvent.

Sampling of the floral scent was conducted in the two flowering seasons, namely, March–April of 2014 and 2015. The plant individuals were transferred from common garden to growth chamber (Biotron; NK System, Osaka, Japan) $>$14 h before sampling. The growth chamber was set at 10℃ with 80% moisture from 1600 to 1000 and at 20℃ with 70% moisture from 1000 to 1600.

For floral scent sampling, 10 fully-bloomed flowers, if available, were removed from the flowering stems of an individual plant and placed in a 4 mL glass vial, the top of which was covered with aluminum foil. In the case only less than 10 fully bloomed flowers were available, three flowers were sampled in the same way and placed in a 1.5 mL silicon-capped glass vial (Shimadzu, Kyoto, Japan). For the analytical standard, 100 μL of each of them was placed in the 1.5 mL silicon-capped glass vial, and samplings and analyses were conducted with two replicates for each concentration.

SPMS needle was inserted in the glass vial containing the flowers, and the 100 μm polydimethylsiloxane (PDMS) fiber (Supelco, Bellefonte PA, USA) was exposed to the headspace for 30 min. The sampling room temperature was set to 24–26℃. The sampled SPMS fiber was immediately analyzed with gas chromatography (GC). As the control, ambient air and the headspace of chopped vegetative parts of the plants were also sampled, while no GC signals were detected from these samples.

GC and data analyses

For analyses of the floral volatile compositions, GC was performed with Shimadzu GC-17A (Shimadzu) equipped with a flame ionization detector (FID) and an InertCap CHIRAMIX capillary column (30 m × 0.25 mm; film thickness, 0.25 μm; GL science Inc., Tokyo, Japan). Helium was used as the carrier gas at a velocity of 30 cm s^{-1}, and the injector temperature was 230℃. The injector was operated in the splitless mode for 1 min. The oven temperature was programmed to the following sequence: 40℃ for 5 min, followed by an increase of 2℃/min to 180℃, at which the oven was held for 15 min. Signals were detected by flame ionization detector, and the peak area was used for quantification of each signal.

The chromatogram peaks were identified by comparing the chromatograms of the parental species (*Mitella furusei* and *M. pauciflora*), of which the profiles of the scent compounds were already available (Okamoto *et al.*, 2015). Where available, the authentic compounds were also examined ((*RS*)-linalool, (*R*)-linalool, and *β*-caryophyllene; Wako, Tokyo, Japan or Sigma-Aldrich, Tokyo, Japan) using the GC with the same settings to further confirm the identity of each peak.

All statistical analyses were conducted using R (R Development Core Team, 2011).

Results

Contents of the floral scent

As expected, the floral scents of the F2 plants were variable among samples but the contents were only those observed in the parental species. Linalool, *β*-caryophyllene, and lilac aldehyde (tentative identification) are found to be the most constantly detected compounds across the samples. Because in the present study, a GC column capable of resolving enantiomers was used, linalool was further dissected into (*R*)- and (*S*)-linalool. Accordingly, in the present study, I selected only (*R*)- and (*S*)-linalool, lilac aldehyde, and *β*-caryophyllene as the target of the quantification.

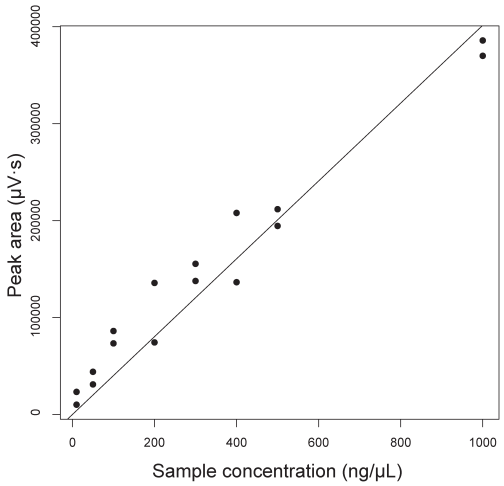

Fig. 1. The relationship between the concentrations of the linalool analytical standards and the GC peak area values obtained by the present method.

Variability and reproducibility of the floral scent phenotypes

Using the analytical standards, I obtained a reference data for roughly quantifying the amount of linalool in the sample. As expected, the peak areas were linearly correlated with the linalool concentration of the analytical standards up to 1000 μg/μL (Fig. 1). From this reference data, a unit (1 μV·s) of the peak areas of (*R*)-linalool and (*S*)-linalool in the samples was roughly calculated as equivalent to the headspace of the analytical standards with 2.5×10^{-3} ng/μL in concentration, respectively.

Floral scent phenotypes were variable across the samples, ranging from zero (no signal) to 94882 μV·s for (*R*)-linalool, from zero to 382646 μV·s for (*S*)-linalool, from zero to 10551 μV·s for lilac aldehyde, and from zero to 193738 μV·s for *β*-caryophyllene (Table 1). The

Table 1. The floral scent phenotypes for 29 F2 individuals across two flowering seasons in the four principal volatile compounds (unit: μV·s)

F2 plant ID	*R*-Linalool (2014)	*R*-Linalool (2015)	*S*-Linalool (2014)	*S*-Linalool (2015)	lilac aldehyde* (2014)	lilac aldehyde* (2015)	*β*-Caryo-phyllene (2014)	*β*-Caryo-phyllene (2015)
IF2-003	10513	6237	389	376	0	0	107275	108659
IF2-013	15450	21863	3983	5712	1364	2376	0	0
IF2-019	7306	16713	2589	3085	2153	2493	90023	147725
IF2-022	19154	28499	6725	11544	4020	1598	1036	2762
IF2-023	4954	5342	1900	1864	977	712	0	946
IF2-027	17229	29704	17235	41442	1945	548	20571	74555
IF2-034	209	0	2226	1095	0	227	54851	109005
IF2-039	16629	7243	12296	9948	2088	1042	82934	54824
IF2-041	47212	94882	222589	181838	0	0	0	0
IF2-043	31089	19842	154690	242879	0	0	0	0
IF2-046	60503	70101	98391	246036	0	0	75370	103056
IF2-048	0	0	1505	981	0	0	2937	53754
IF2-054	7763	12302	955	7715	681	568	1406	0
IF2-056	12891	39347	2526	11061	1137	785	42059	64009
IF2-059	9900	11179	4053	9351	1712	1969	52458	29309
IF2-060	246	614	185071	250290	0	0	71831	165089
IF2-061	6465	4829	55	0	0	0	149252	104745
IF2-074	0	767	240581	257592	0	0	27143	120243
IF2-099	10055	13931	1727	5445	0	0	97881	144352
IF2-100	0	0	0	0	0	0	26838	166911
IF2-108	30937	32457	237146	382646	0	0	66768	62957
IF2-110	10225	8284	103	0	0	0	86870	123962
IF2-128	6690	5593	3009	3743	0	0	98472	81298
IF2-141	8000	26336	7487	4010	4456	2289	30656	137740
IF2-152	9307	17020	0	0	0	0	193738	132166
IF2-171	10138	6689	9031	7742	1816	10551	98304	33912
IF2-194	12277	4431	7311	5252	272	4141	0	0
IF2-246	2579	10590	3298	4547	256	1434	16835	122181
IF2-260	349	700	6968	13539	2681	6478	92453	94222

*Tentative identification based on the published data of the parental species (Okamoto *et al.*, 2015)

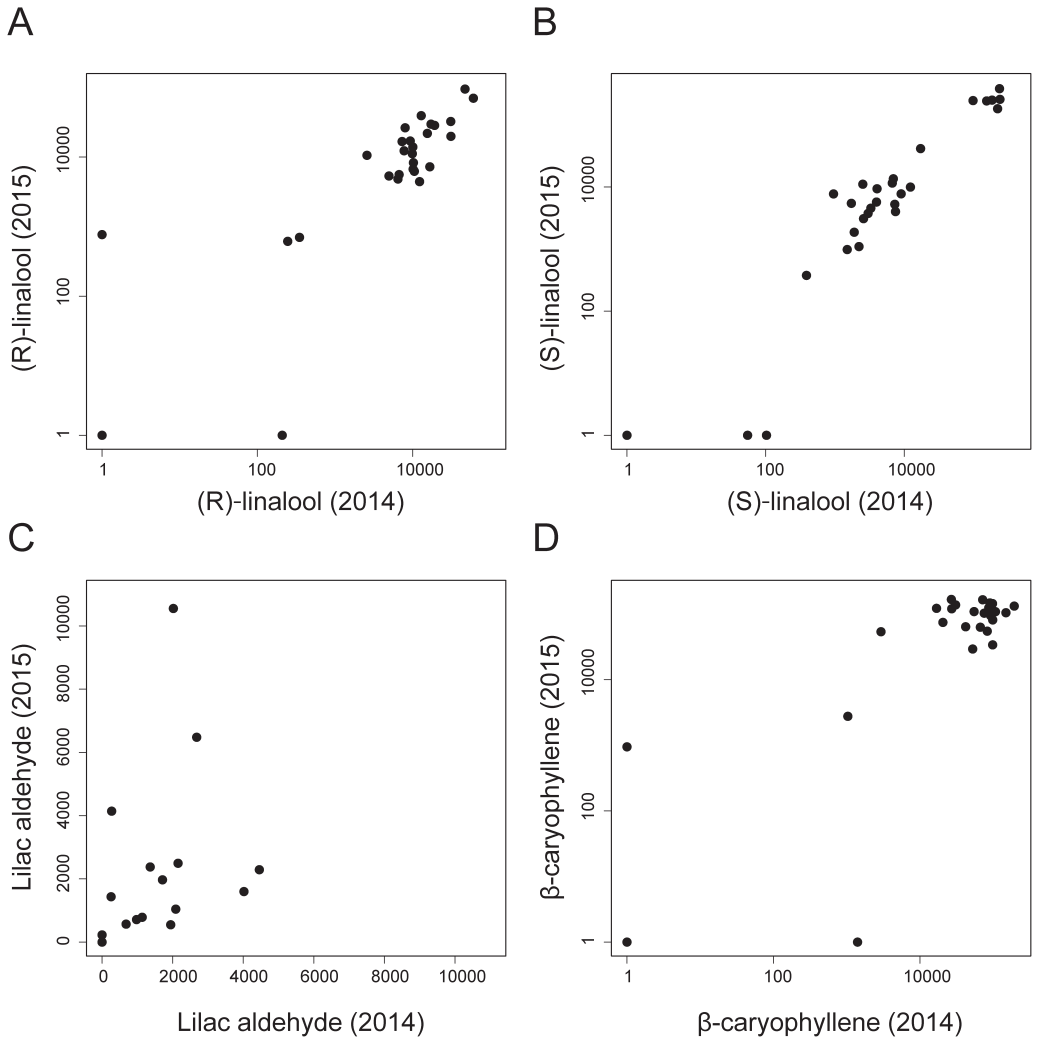

Fig. 2. The patterns of correlations of the floral scent phenotypes between 2014 and 2015 in the four volatile compounds. A: (*R*)-linalool, B: (*S*)-linalool, C: lilac aldehyde (tentative identification), D: *β*-caryophyllene. For A, B and D, the both axis are log-transformed and the zero values are plotted as 1.

floral scent phenotypes quantified in the two flowering seasons were modestly to strongly correlated with each other for all the four compounds (Fig. 2; Spearman's rank correlation test; $p < 9.8 \times 10^{-8}$, $\rho = 0.81$ for (*R*)-linalool, $p < 1.1 \times 10^{-10}$, $\rho = 0.89$ for (*S*)-linalool, $p < 8.4 \times 10^{-11}$, $\rho = 0.89$ for lilac aldehyde, and $p < 9.2 \times 10^{-4}$, $\rho = 0.58$ for *β*-caryophyllene). Simple linear regression analyses (intercept was set to zero) were also all highly significant ($p < 0.001$) and showed that the scent phenotype quantified in 2014 well predicts that quantified in 2015 for all the four compounds ($y = 1.3x$, $r^2 = 0.86$ for (*R*)-linalool, $y = 1.3x$, $r^2 = 0.92$ for (*S*)-linalool, $y = 1.1x$, $r^2 = 0.39$ for lilac aldehyde, and $y = 1.1x$, $r^2 = 0.67$ for *β*-caryophyllene).

Discussion

In this study, I successfully established a standard methodology for consistently and quantitatively phenotyping the floral scent traits of

Mitella. The reference data using the analytical standards indicated that the values obtained with the present methodology is proportional to the amount of volatiles in the headspace, at least for linalool within the concentration range examined in the present study. The obtained values for the individual volatile compounds were variable among F2 individuals but stable across the two flowering seasons within each individual (Fig. 2). These observations indicate that the present phenotyping method is reliable enough to apply it to genetic analyses and any comparative analyses on the scent traits. Moreover, the present study led to another important conclusion, that is, the traits studied in the present study are likely under the strong control of the plant genotype along with the other phenotypic traits already studied in the previous study (Okuyama and Akashi, 2013). Because the parental species of the F2 hybrids were divergent in the floral scent traits and associated pollinators they relied on (*M. furusei* and *M. pauciflora*; Okamoto *et al.*, 2015), these findings would be the strong basis to pursue the genetic study identifying the genetic basis of floral scent traits and associated reproductive isolation mechanisms in the plant lineage.

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