Metabolic Regulation of Floral Scent in Petunia axillaris Lines: Biosynthetic Relationship between Dihydroconiferyl Acetate and iso-Eugenol

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Aromatic scent-related compounds in flowers were comprehensively analyzed by high-performance liquid chromatography (HPLC) based on their absorption spectra to understand regulation of metabolism leading to floral scent diversity in Petunia axillaris lines. An unknown compound occurring at similar levels to scent compounds in some plant lines was identified to be dihydroconiferyl acetate. Based on the structure, dihydroconiferyl acetate is likely to be a biosynthetically closely related compound to aromatic scent compounds, especially iso-eugenol. Similar time-course changes of the concentrations suggest that the metabolism of dihydroconiferyl acetate is underlaid by the similar regulation to aromatic scent compounds. Dihydroconiferyl acetate and iso-eugenol occurred almost exclusively, implying that metabolism of the common precursors to each compound is selectively regulated in these plants. The branching of the biosynthetic pathway into dihydroconiferyl acetate and iso-eugenol is probably one of regulatory steps leading to scent diversity in P. axillaris lines.

Key words: floral scent; iso-eugenol; Petunia axillaris; volatile benzenoid/phenylpropanoid

Petunia axillaris (Lam.) Britton, Stern, and Poggenb. is thought to be one of the parental species of the garden petunia. It is widely distributed in temperate South America. 1) This species is classified into three allopatric infraspecific taxa: subsp. axillaris, subsp. parodi (Steere) Cabrera, and subsp. subandina T. Ando. 2) P. axillaris flowers emit a strong, musty scent at nighttime only. Among P. axillaris are various lines emitting scents that are different. 3) We assumed that these genetically close plants can become a powerful tool for understanding the generating mechanism of floral scent diversity. A successful study was carried out in the genus Clarkia; a linalool synthase gene was cloned by comparing expressed genes between the scented C. breweri and the scentless C. concinna. 4–6) Taking a different view of regulation on evaporation process from floral tissue, both endogenous and emitted components of several P. axillaris plant lines were analyzed. 7) The endogenous scent components were qualitatively and quantitatively various due to differences in regulation of production, whereas the qualitative variation was reduced in the emitted components, where lower-boiling-point compounds predominated. This phenomenon suggests that emission is primarily a physical phenomenon; hence, diversity of floral scent properties is generated by variation in metabolism through evaporation. In order to determine the diversity of floral scents, biosynthetic regulation to generate the qualitative and quantitative variation of endogenous components should primarily be studied.

Generally, floral scents are defined as volatile compounds possessing fragrance among secondary metabolites biosynthesized in flowers. 8) Aromatic scent compounds are biosynthesized from L-phenylalanine through many scentless or nonvolatile intermediates. 9) A comprehensive analysis of biosynthetically related compounds, even if they are scentless compounds, should yield important information for understanding the regulation of scent biosynthesis. In our sequential study,
a putative aromatic compound was detected in petals of several *P. axillaris* lines at levels similar or higher to other aromatic scent compounds. We discuss the metabolic relationship of this compound to aromatic scent compounds, especially to *iso*-eugenol.

**Materials and Methods**

*Plant materials.* Plants of *Petunia axillaris* subsp. *axillaris*, subsp. *parodii*, and subsp. *subandina* were raised from seeds collected from several natural populations in Uruguay, Brazil, and Argentina. The same 13 individuals chosen in our previous study were used for experiments: B58AXI9, B1320AXI10, B1320AXI13, U242AXI4, U1AXI, B298PAR7, U220PAR9, U200PAR10, U238PAR11, U238PAR15, A130SUB9, A139SUB2, A203SUB8.7) The initial letter and number indicate the herbarium specimen code, with a letter indicating the country of the origin: A (Argentina), B (Brazil), U (Uruguay). The subspecies abbreviation and the individual code are indicated by the following letters and the individual code (AXI, subsp. *axillaris*; PAR, subsp. *parodii*; SUB, subsp. *subandina*). These lines were propagated vegetatively in a greenhouse 2 months before the experiments. Just before the experiments, the plants were acclimated for at least 1 week in a growth chamber at a constant temperature of 25°C.

**Extraction and preparation of endogenous benzenoid compounds.** Flower tissues excluding the calyx were frozen in liquid nitrogen and ground in a mortar. The resulting powder was extracted twice with pentane (5 ml each time) in a microwave oven (700 W) for 20 s. Anisole or ethyl decanoate (20 μg each time) was added as an internal standard for HPLC analysis and GC analysis respectively. The extract was dehydrated with anhydrous sodium sulfate and concentrated at 40°C in a water bath.

**GC–MS and GC analysis of scent-related compounds.** Capillary gas chromatography–mass spectrometry (GC–MS) was performed using an Agilent 6890N gas chromatograph coupled to an Agilent 5973N Mass Selective Detector (Agilent Technologies, Wilmington, DE).11) The GC was equipped with a splitless injector and a DB-WAX capillary column (30 m in length, 0.25 mm i.d., and 0.25 μm film thickness). The column oven temperature was kept at 45°C for the first 2 min, then increased by 3°C/min to 220°C, and maintained at 220°C for 10 min. The injection, interface, and ion source temperatures were 250°C, 280°C, and 250°C respectively. Helium was used as the carrier gas. GC analysis was performed using an Agilent 6850 gas chromatograph (Agilent Technologies) monitored by FID. Analytical conditions were the same as for GC–MS, as described above.11) The amounts of the each compound were calculated in comparison with the peak area of the internal standard. The compound identifications were made by NIST02 library search provided with GC–MS software. Identification was substantiated on the basis of mass spectra and retention time by subjecting authentic samples to GC–MS analysis under the same conditions. The unknown compound was eluted at t<sub>R</sub> 64.2 min, and the EI/MS spectrum showed signals at m/z (rel. int.) 224 [M<sup>+</sup>] (65), 164 (69), 149 (36), and 137 (100).

**HPLC analysis of benzenoid compounds.** High performance liquid chromatography (HPLC) was performed using an Agilent 1100 chromatograph (Agilent Technologies). The analytical conditions were as follows: column, Cadenza CD-C18 column (2 mm i.d. x 250 mm, Imtact, Kyoto, Japan); column temperature, 40°C; flow rate, 0.2 ml/min; solvent A, acetonitrile with 0.1% acetic acid (HOAc); solvent B, distilled water with 0.1% HOAc; gradient program, 20% A from 0 to 10 min, 20% to 30% A from 10 to 20 min, 30% to 35% A from 20 to 30 min, 35% to 100% A from 30 to 50 min, 100% A from 50 to 80 min. Detection and concentration measurements of compounds were performed by the absorbance of each compound in comparison with the peak area of the internal standard.

**Isolation and identification of unknown compound.** A putative aromatic compound was extracted under the same conditions as for the endogenous volatiles from petals of U242AXI4. The crude extract was purified by HPLC using an Agilent 1100 chromatograph (Agilent Technologies). Conditions for purification were as follows: column, ODS-4253-D column (10 mm i.d. x 250 mm, Senshu Pak, Tokyo); column temperature, 40°C; flow rate, 1.0 ml/min; solvent A, acetonitrile with 0.1% acetic acid (HOAc); solvent B, distilled water.
with 0.1% HOAc; gradient program, 20% A from 0 to 10 min, 20% to 30% A from 10 to 20 min, 30% to 35% A from 20 to 30 min, 35% to 100% A from 30 to 50 min, 100% A from 50 to 80 min. Developing solvent was collected from 33 to 35 min.

The unknown compound was analyzed by NMR in CD$_3$OD at 400 MHz on a Bruker Biospin ARX400 system. The $^1$H-NMR spectrum showed signals at $\delta_H$ 1.90 (2H, ttd, $J = 6.5, 7.6, 14.9$ Hz, H-8), 2.02 (3H, s, methyl protons of acetyl group), 2.59 (2H, $t$, $J = 7.6$ Hz, H-7), 3.82 (3H, s, methyl protons of methoxy group), 4.04 (2H, t, $J = 6.5$ Hz, H-9), 6.60 (1H, dd, $J = 1.7, 8.0$ Hz, H-6), 6.69 (1H, d, $J = 8.0$ Hz, H-5), and 6.75 (1H, d, $J = 1.7$ Hz, H-2). The $^{13}$C-NMR spectrum showed signals at $\delta_C$ 20.8 (methyl carbon of acetyl group), 31.7 (C-8), 32.6 (C-7), 56.3 (methyl carbon of methoxy group), 65.0 (C-9), 113.1 (C-2), 116.1 (C-5), 121.8 (C-6), 134.1 (C-1), 145.7 (C-4), 148.9 (C-3), and 173.1 (carbonyl carbon of acetyl group).

Dihydroconiferyl alcohol was purchased from Funakoshi Co., Ltd. (Tokyo), and analyzed by NMR under the same conditions. The $^1$H-NMR spectrum showed signals at $\delta_H$ 1.79 (2H, ttd, $J = 6.8, 7.7, 15.1$ Hz, H-8), 2.58 (2H, $t$, $J = 7.7$ Hz, H-7), 3.54 (2H, $t$, $J = 6.8$ Hz, H-9), 3.82 (3H, s, methyl protons of methoxy group), 6.61 (1H, dd, $J = 1.8, 8.0$ Hz, H-6), 6.68 (1H, d, $J = 8.0$ Hz, H-5), and 6.76 (1H, d, $J = 1.8$ Hz, H-2).

The $^{13}$C-NMR spectrum showed signals at $\delta_C$ 32.6 (C-7), 35.7 (C-8), 56.3 (methyl carbon of methoxy group), 62.3 (C-9), 113.1 (C-2), 116.1 (C-5), 121.8 (C-6), 134.9 (C-1), 145.5 (C-4), and 148.8 (C-3).

**Result**

**Structural determination of unknown compound**

An unknown compound showing three absorption maxima, at 210, 230, and 282 nm, similar to the spectra properties of iso-eugenol ($\lambda_{\text{max}}$: 214, 260, and 300 nm), was eluted in a fraction of I$_R$ 33–35 min by ODS-HPLC (Fig. 1). Dominancy in the chromatogram and variation in peak size among lines prompted us to further analysis of this compound. A molecular ion peak at $m/z$ 224 (M$^+$) and a fragment ion peak at $m/z$ 164 (M$^+$ – 60) suggested the putative molecular formula of C$_{12}$H$_{16}$O$_4$ and the presence of an acetyl group. The presence of similar signals with dihydroconiferyl alcohol in both $^{13}$C-NMR and $^1$H-NMR spectra and additional signals of the acetyl group ($\delta_H$ 2.02 and $\delta_C$ 20.8 and 173.1) suggested that this compound was an acetyl derivative of dihydroconiferyl alcohol. Lower field shifts of the H-9 signal ($\delta_H$ 4.04 from 3.54) and the C-9 signal ($\delta_C$ 65.0 from 62.3) in this compound suggested binding of the acetyl group to O-9 of dihydroconiferyl alcohol. Finally, the structure of this unknown compound was determined to be dihydroconiferyl acetate. We did not detect any fragrance from this aromatic compound.

**Time-course of endogenous levels of scent compounds and dihydroconiferyl acetate**

The time-courses change of endogenous levels of dihydroconiferyl acetate and scent compounds were investigated in the U242AXI4 line (Fig. 2). Among scent compounds, data on methyl benzoate, which is universally observed in P. axillaris, and iso-eugenol, which has structural similarity to dihydroconiferyl acetate, are representative shown in Fig. 2. Neither scent compounds nor dihydroconiferyl acetate was detected in flowers before anthesis. The concentration of dihydroconiferyl acetate started to increase after anthesis at about 12:00 and reached the maximum level at 3:00 of the first night (100.6 nmol/0.1 g f.w.). After 3:00, the concentration decreased to the minimum level at 15:00. The increase and decrease in nighttime and daytime respectively was maintained during flower life.
that of other scent compounds. 7) The volatility of the compound was extremely low in comparison with flowering day to 17:00 on the next day. The volatility of acetate was detected for 24 h from 17:00 on the acetate. About 6.5 nmol/0.1 g f.w. of dihydroconiferyl mum levels at noon.

reaching their maximum levels at midnight and mini-

was basically synchronous with those of the major scent coniferyl acetate concentration in the U242AXI4 line was also maintained. The time-course of the dihydro-

for at least 4 d, until which time the vividness of petals was also maintained. The time-course of the dihydroconiferyl acetate concentration in the U242AXI4 line was basically synchronous with those of the major scent compounds, including methyl benzoate and iso-eugenol, reaching their maximum levels at midnight and minimum levels at noon.

A flower of the U242AXI4 line (about 0.4 g/flower) was used for headspace collection of dihydroconiferyl acetate. About 6.5 nmol/0.1 g f.w. of dihydroconiferyl acetate was detected for 24 h from 17:00 on the flowering day to 17:00 on the next day. The volatility of the compound was extremely low in comparison with that of other scent compounds. 7)

Variation in composition of scent-related compounds

The endogenous concentration of scent compounds increased during nighttime in U1AXI. 11) Earlier we found that endogenous concentration is related to emitted amount in each scent compound. 7,11) The sensory strength of floral scent increased during nighttime in other P. axillaris lines (data not shown), and hence we thought that endogenous concentrations of scent compounds probably increased during nighttime as well as dihydroconiferyl acetate in each line. We compared the endogenous concentrations of dihydroco-

niferyl acetate and iso-eugenol among lines using the values at 24:00.

Based on the absolute amount of dihydroconiferyl acetate and iso-eugenol, and the abundance ratio of them, 13 lines were classified into three groups (Table 1). Dihydroconiferyl acetate with a great major-

ity (> 85% of sum; > 30 nmol/0.1 g f.w.) was detected in eight lines belonging to each subspecies of parodii, axillaris, and subandina, and so these plants were assigned to group 1. The concentration ranged from 33.9 nmol/0.1 g f.w. in A203SUB8 to 77.8 nmol/0.1 g f.w. in U238PAR15. The concentrations of iso-eugenol in group 1 were much lower than those of dihydroco-

niferyl acetate (less than 15%); many lines contained iso-eugenol at less than 1.0 nmol/0.1 g f.w. The remaining five lines did not accumulate dihydroconiferyl acetate, where U1AXI and B58AXI9, which were assigned to group 2, contained levels of iso-eugenol comparable to those of dihydroconiferyl acetate in group 1 plants, and A139SUB2, B1320AXI10, and B1320AXI13, which were assigned to group 3, contained low concentrations of both iso-eugenol and dihydroconiferyl acetate (less than 10 nmol/0.1 g f.w.). trans-Cinnamic acid (tR 29.3 min, obtained by HPLC analysis of the standard) and p-coumaric acid (tR 8.5 min), caffeic acid (tR 5.6 min), and ferulic acid (tR 9.4 min), which are putative biosynthetic precursors of iso-
eugenol and dihydroconiferyl acetate, were analyzed by HPLC, but these compounds were not detected in any line (Fig. 1).

Fig. 2. Time-Courses of Endogenous Levels of Dihydroconiferyl Acetate (A), Methyl Benzoate (B), and iso-Eugenol (C) in P. axillaris Line U242AXI4.

Measurements were taken over 3-h periods, and mean values ± SE (n = 3) are shown by vertical bars.

Table 1. Concentrations of Dihydroconiferyl Acetate and iso-Eugenol in 13 Lines of P. axillaris

<table>
<thead>
<tr>
<th>Group</th>
<th>Specimen code</th>
<th>Dihydroconiferyl acetate (nmol/0.1 g.f.w.)</th>
<th>iso-Eugenol (nmol/0.1 g.f.w.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A130SUB9</td>
<td>35.8 ± 1.2*</td>
<td>n.d.*</td>
</tr>
<tr>
<td></td>
<td>A203SUB8</td>
<td>33.9 ± 6.8</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>B298PAR7</td>
<td>53.4 ± 6.6</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>U220PAR9</td>
<td>52.8 ± 0.7</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>U220PAR10</td>
<td>62.9 ± 5.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>U238PAR11</td>
<td>67.5 ± 11.7</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>U238PAR15</td>
<td>77.8 ± 8.1</td>
<td>10.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>U242AXI4</td>
<td>60.0 ± 7.4</td>
<td>9.2 ± 1.6</td>
</tr>
<tr>
<td>2</td>
<td>B58AXI9</td>
<td>n.d.</td>
<td>33.2 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>U1AXI</td>
<td>n.d.</td>
<td>58.2 ± 11.0</td>
</tr>
<tr>
<td>3</td>
<td>A139SUB2</td>
<td>n.d.</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>B1320AXI10</td>
<td>n.d.</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>B1320AXI13</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

*From Kondo et al. 7)

Means ± SE (n = 3).

*Not detected.
Discussion

An unknown compound detected in flower tissues of several *P. axillaris* lines was determined to be dihydroconiferyl acetate. Based on structure, dihydroconiferyl acetate is thought to be biosynthesized from coniferyl alcohol through dihydroconiferyl alcohol or coniferyl acetate. Coniferyl alcohol is biosynthesized by the C$_6$–C$_3$ pathway, which is successively composed of L-phenylalanine, trans-cinnamic acid, p-coumaric acid, caffeic acid, and ferulic acid, so that dihydroconiferyl acetate is probably a compound biosynthesized in the C$_6$–C$_3$ pathway. Koeduka et al. lately demonstrated that *iso*-eugenol is biosynthesized from coniferyl acetate as a direct precursor in *Petunia*. Dihydroconiferyl acetate appears to be biosynthetically a compound closely related to *iso*-eugenol (Fig. 3). Dihydroeugenol, likely to be a deacetylation product of dihydroconiferyl acetate, was not detected in either emitted or endogenous components (data not shown). Other C$_6$–C$_3$ scent compounds, e.g., vanillin and eugenol, were detected in only a few lines in extremely small amounts.

The concentrations of aromatic scent compounds were kept at low levels before flowering in tissues of U242AXI4 (Fig. 2). After flowering, concentrations of aromatic scent compounds started to increase and showed time-course changes increasing during nighttime and decreasing during daytime. These profiles appear to be characteristics of aromatic scent compounds in *Petunia*. The same time-course changes was found in the concentration of dihydroconiferyl acetate, suggesting that the metabolism of dihydroconiferyl acetate is underlain by a regulation similar to that leading to the time-course change in aromatic scent compounds. The emitted amount of dihydroconiferyl acetate was much less than the reduction in the endogenous level from the maximum to the minimum, and cannot make up the decrease. Hence the decrease in dihydroconiferyl acetate during daytime is mainly attributable to the conversion to other molecules. We have indicated that this unknown mechanism operates upon decrease in scent compounds that have relatively high volatility. Since dihydroconiferyl acetate accumulates highly in tissues and is scarcely lost by evaporation, this is a suitable example to discuss this matter.

A130SUB9 and other lines that belong to group 1 contained higher concentrations of dihydroconiferyl acetate and lower concentrations of *iso*-eugenol, and *vice versa*; U1AXI and B58AXI9, which belong to group 2, contained higher concentrations of *iso*-eugenol and lower concentrations of dihydroconiferyl acetate. Dihydroconiferyl acetate and *iso*-eugenol appeared to occur semi-exclusively in these groups, suggesting that metabolism of the common precursors to each compound is selectively regulated in these plants. In plant lines of both group 1 and group 2, the operation of the C$_6$–C$_3$ pathway showed similar activity until the branching point into *iso*-eugenol and dihydroconiferyl acetate. This indicates that profiling of scentless com-
pounds can give metabolic information on scent compounds. As for plant lines belonging to group 3, the regulatory steps leading to the decrease in iso-eugenol and dihydroconiferyl acetate have not been determined, because no intermediates of the C₆–C₃ pathway were detected.

We have discussed the operation, regulation, and branching of the biosynthetic pathway based on the endogenous profile of dihydroconiferyl acetate, and the branching of the C₆–C₃ pathway into dihydroconiferyl acetate and iso-eugenol which is probably one of the regulatory steps leading to scent diversity in *P. axillaris* lines. This study argues that comprehensive analysis of biosynthetically related compounds is an effective method of understanding the metabolic regulation of scent compounds. This viewpoint will be applied to studies on other horticulturally important plants.

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**References**