Biogenesis of 2-Phenylethanol in Rose Flowers: Incorporation of \([^{2}H_{8}]L\)-Phenylalanine into 2-Phenylethanol and its \(\beta\)-D-Glucopyranoside during the Flower Opening of \(R\)osa ‘Hoh-Jun’ and \(R\)osa \(d\)amascena Mill.

Shuzo WATANABE,1 Kentaro HAYASHI,2 Kensuke YAGI,2 Tatsuo ASAI,3 Hazel MACTAVISH,4† Joanne PICONE,4 Colin TURNBULL,4 and Naoharu WATANABE2,∗

1The United Graduate School of Agricultural Science, Gifu University (Shizuoka University), 836 Ohya, Shizuoka 422-8529, Japan
2Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan
3University Farm, Faculty of Agriculture, Shizuoka University, 63 Kariyado, Fujieda, Shizuoka 426-0001, Japan
4Department of Agricultural Sciences, Imperial College at Wye, University of London, Wye, Ashford, Kent TN25 5AH, U.K.

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To clarify the biosynthetic pathway to 2-phenylethanol (2), the deuterium-labeled putative precursor, \([^{2}H_{8}]L\)-phenylalanine (\(^{2}H_{8}-1\)), was fed to the flowers of \(R\)osa ‘Hoh-Jun’ and \(R\). \(d\)amascena \(M\)ill. throughout maturation, ceasing feeding at the commencement of petal unfurling and at full bloom. Based on GC-MS analyses, \([^{2}H_{4}]1\) was incorporated into both 2 and 2-phenylethyl \(\beta\)-D-glucopyranoside (3) when the flowers were fed until full bloom, whereas no such incorporation into 2 was apparent when feeding was ceased earlier. In both species of rose, the labeling pattern for 2 was almost identical to that for 3, and indicated the presence of \([^{2}H_{6}]1\), \([^{2}H_{7}]1\)- and \([^{2}H_{8}]1\)-2, and \([^{2}H_{6}]1\), \([^{2}H_{7}]1\)- and \([^{2}H_{8}]1\)-3. This may be ascribed to the equilibrium between 2 and 3. The labeling pattern for 2 and 3 also indicated that these compounds were produced from 1 via several routes, the route involving phenylpyruvic acid being the major one.

Key words: rose flower; scent formation; biogenesis; 2-phenylethanol; 2-phenylethyl \(\beta\)-D-glucopyranoside

We have recently shown that the glycoconjugates of monoterpene alcoholic aromas, such as citronellol and geraniol, were not involved in the scent formation in flowers of \(R\)osa \(d\)amascena \(M\)ill.,1) which is an important cultivar for the production of essential oil. However, several alcoholic aromas emitted from flowers have been reported to be formed from glycosidic precursors.2–6) In contrast to monoterpene alcohols, only a trace amount of 2-phenylethanol (2) has been detected in \(R\). \(d\)amascena before the commencement of petal unfurling (between stages 1 and 2, refer to the \(P\)lant materials section), whereas 2-phenylethyl \(\beta\)-D-glucopyranoside (3) was predominantly accumulated.1,7) After stage 2, the flowers emitted a high aroma level of 2, while the level of 3 declined.1) The \(\beta\)-glucosidase activity was also elevated dramatically during the period.1) Thus, 2 emitted during the process of flower opening seemed to be formed from glycoconjugates such as 3 by the action of \(\beta\)-glucosidase (route e in Fig. 1). In fact, \([^{1}\text{-}H]3\) fed to immature flowers was converted to \([^{1}\text{-}H]2\) by the full-bloom stage,8) implying the potential role of 3 in rose

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Fig. 1. Hypothetical Biogenetic Pathway for 2-Phenylethanol (2) and 2-Phenylethyl \(\beta\)-D-Glucopyranoside (3) from 1-Phenylalanine (1).
We performed further investigations to elucidate the biosynthetic routes to 2 and 3, and have developed a method to infiltrate rose flowers with the putative precursors.

In yeast, 2 has been reported to be biosynthesized from the corresponding α-keto acid, phenylpyruvic acid (5), by decarboxylation and subsequent reduction. Compound 5 can be derived from L-phenylalanine (1) by deamination (route b in Fig. 1). In plant tissues, 1 has been reported to be transformed to 2-phenylethylamine (4), 5 or trans-cinnamic acid (6). Thus, 1 can be expected to lead to 2 and/or 3 via 4, 5 and/or 6. Compound 3 can be derived from 2 by the action of glucosyltransferase (route d). Alternatively, 3 can also be derived from phenyllactic acid β-D-glucopyranoside by decarboxylation via a pathway (route f) not involving 2.

R. ‘Hoh-Jun’ is a cultivar which also emits 2 as a major scent compound and accumulates high levels of 3 as a major glycoside of 2 in flowers at stages 1 and 2.9 We therefore undertook a preliminary experiment by feeding [H₈]-1 to an intact immature flower of R. ‘Hoh-Jun’ to see if it could be incorporated into 2 and/or 3. We also fed higher concentrations of [H₈]-1 to the flowers of R. ‘Hoh-Jun’ and R. damascena Mill. throughout the period starting from immature flowers (stage 1) prior to petal unfurling (4 days after stage 1), petals fully retracted, outer petal whorl beginning to loosen; stage 3, outer petal whorl opened, inner petal whorl beginning to loosen; stage 4, inner petal whorl opened but reproductive organs not yet visible; stage 5, full bloom flower, inner and outer whorls open, reproductive organs visible, 6hr (R. damascena) or 2 days (R. ‘Hoh-Jun’) after stage 2.

Materials and Methods

Chemicals and biochemicals. 2,3,3, 2'-3',4',5',6'-[H₈]-Phenylalanine ([H₈]-1, 98 atom % ²H, Aldrich) was used. A comparison of the EI-MS data for the methyl esters of [H₈]-1 and [H₈]-1 in [H₈]-1 was less than 0.5% based on the relative ion intensities at m/z 154/m/z 155/m/z 156 ([M-OCH3]⁻ for [H₈]-1)/m/z 157=1.07/1.06/1.30/2.96 and at m/z 146/m/z 147/m/z 148 ([M-OCH₃]⁻ for 1)/m/z 149=1.01/1.01/1.31/3.00. 1,1,2',2',3',4',5',6'-[H₈]-2 was synthesized by reduction with 9-BBN from 1,1,2',2',3', 4',5',6'-[H₈]-styrene (98 atom % ²H, Cambridge Isotope Laboratories, MA, U.S.A.) as a control for the GC-MS analysis. The details will be reported elsewhere. β-Glucosidase (EC 3.2.1.1, 500 units/mg from almond, Sigma) and naringinase (300 units/mg from Penicillium decumbens, Sigma) were used for enzymatic hydrolysis of the glycoconjugated volatile compounds.

Preliminary feeding experiments of [H₈]-phenylalanine (1) into immature flowers of Rosa ‘Hoh-Jun’ from stage 1 until stage 2: Experiment 1. Incorporation studies in October 2000 were conducted on immature flowers at stage 1 with intact plants which would open four days later under natural conditions. A thin needle with a cotton thread (50 mm in length) was inserted at 10 a.m. on day 0 through the top of the ovary, just above the ovules. This thread acted as a wick enabling the absorption of an aqueous solution of [H₈]-1 (1.1 mm, 1.5 ml) from an Eppendorf tube attached to the stem. Once every day at 10 a.m., 50 μl of water was applied to ensure that the thread remained wet. The total amount of [H₈]-1 absorbed by the plant was not evaluated. On day 5, this feeding was stopped, and five treated flower heads at stage 2 were detached just below the ovary of the flower.

Feeding of [H₈]-phenylalanine (1) to immature flowers of Rosa ‘Hoh-Jun’ and Rosa damascena Mill. from stage 1 to stage 5: Experiment 2. In May, 2001, [H₈]-1 was fed to the flowers of R. damascena Mill. and R. ‘Hoh-Jun’ by the method just described. [H₈]-1 (3.3 mm, 1.5 ml) was fed to immature flowers at stage 1 of each species until full bloom at stage 5 (6 and 4 days for R. ‘Hoh-Jun’ and R. damascena, respectively). The flowers were detached immediately after feeding and treated as described next to extract 2 and 3.

Extraction of the volatile compounds and glycoconjugates. The flower petals were immediately frozen in liquid nitrogen, and a portion (equivalent to one flower head) of each sample was extracted twice with pentane (20 ml each) and then twice with EtOAc (20 ml each) in a microwave oven according
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**Results and Discussion**

As shown in Fig. 2, [H$_8^2$]-I could be expected to lead to four types of 2-phenylethanol (2a–2d) via 4, 5 and/or 6, with different labeling patterns.

In Experiment 1, [H$_8^2$]-I was fed to immature flowers of *R. Hoh-Jun* at stage 1 until stage 2. GC-MS data for the pentane-CH$_2$Cl$_2$ extract from an enzymatic hydrolysate of the EtOAc fraction showed an ion peak at $t_R = 33.47$ min for 2, with a series of ion peaks of $m/z$ 122 (36%, M$^+$ for C$_8$H$_{10}$O), 91 (100%, C$_7$H$_7^+$), and 65 (17%, C$_5$H$_5^+$), together with a peak at $t_R = 33.36$ min with the ion peaks at $m/z$ 129 (34%, M$^+$ for C$_9$D$_7$H$_3$O), 98 (100%, C$_7$D$_7^+$), and 70 (18%, C$_5$D$_5^+$). This pattern suggests that the predominant labeled version of 2 was 2b which had a molecular ion at $m/z$ 129 and a predicted fragment ion at $m/z$ 98 (Fig. 3). It was thus confirmed that [H$_8^2$]-I had been incorporated as the aglycone part of 3.

Figure 3C shows several ion intensities different from those of 2 (Fig. 3B); for example, the ratio of 9/34 for $m/z$ 130/129 in Fig. 3C was higher than that of 3/36 for $m/z$ 123/122 in Fig. 3B, while the ratio was 100/28 for $m/z$ 98/97 in Fig. 3C, but 100/2 for $m/z$ 91/90 in Fig. 3B. The enrichment of $m/z$ 130 can be most simply ascribed to the detection of [H$_8^3$]-2 (2a) which was predicted to show...
Fig. 3. GC-MS Analyses of the Pentane-dichloromethane Extract from the Enzymatic Hydrolysate of the Glycosidic Fraction of Rosa 'Hoh-Jun' Fed with [1H,3H]-Phenylalanine ([1H,3H]-1).
(A) Total ion trace, where 2-phenylethanol (2) and [1H,3H]-2 (n = 6–8) were detected.
(B) MS for 2.
(C) MS for [1H,3H]-2 (n = 6–8).

a molecular ion at m/z 130. Furthermore, the higher ion intensity at m/z 97 in Fig. 3C indicates the presence of [1H,3H]-2 (2c) and/or [1H,3H]-2 (2d) which showed a fragment ion at m/z 97.

The deuterated compounds, [1H,3H]-2 (n = 6–8), were detected as a single peak at tR = 33.36 min, because the isotope effects among these compounds were not large enough to allow their resolution as different tR's under the GC conditions used. This observation is substantiated by the fact that authentic 1,1,2,2,3,3',4',5',6'-[1H,3H]-2 gave MS data with ion peaks at m/z 130 (34%), M+ for C9H9D5O and m/z 131(3%, [M + 1]+) at the same tR = 33.35 min, with a similar pattern to that of 2 in the molecular ion cluster at m/z 122 (36%) and m/z 123 (3%).

In Experiment 1, deuterium labeled 2 was only detected as the glycosidic form in the EtOAc fraction, and not in the initial pentane extract which would have contained free 2. Therefore, in Experiment 2, the concentration of [1H,3H]-1 was increased three-fold and feeding was continued until stage 5, when both species emitted 2 together with other volatile compounds. Again, the incorporation of [1H,3H]-1 was confirmed into the aglycone part of 3. The levels of 3 and deuterated 3 detected were 750 and 42 μg/flower head of R. damascena, and 2200 and 430 μg/flower head of R. ‘Hoh-Jun’, respectively. In addition, [1H,3H]-1 was also incorporated into 2 in the pentane extract. The average amounts of 2 and deuterated compounds 2 were 560 and 41 μg/flower head of R. damascena, and 260 and 79 μg/flower head of R. ‘Hoh-Jun’, respectively. These deuterated compounds were predicted to give the characteristic MS spectra as shown in Table 1.

Based on the MS data for 2 and for the aglycone part of 3, the ion intensities at each m/z value were calculated for the predicted labeling patterns of each 2a–2d and then compared with the actual intensities in each mass chromatogram at m/z 130–128 and at m/z 98, 97 as shown in Table 1. The presence of all four compounds 2a–2d, and their corresponding glucosides 3a–3d, were deduced from the following evidence.

Compound 2b ([1H,3H]-2, MW 129) and its glucoside 3b were dominant in all extracts, based on the abundance of m/z 129 and m/z 98. This observation is essentially similar to that of Experiment 1 and suggests that route b was the main path for the biosynthesis of 2 and/or 3. The higher ratio (8.9–9.1/20) of ion intensity for m/z 130/m/z 129 than that for m/z 123/m/z 122 (3/36) strongly suggests the presence of 2a ([1H,3H]-2, MW 130) in the pentane extract from both species. As shown in Fig. 2, it is speculated that 2a would have been enzymatically formed from [1H,3H]-2-phenylethylamine (4) by the action of amine oxidase,13) with subsequent reduction of [1H,3H]phenylacetaldehyde without the loss of deuterium (route a). An ion at m/z 128 was observed at a low level, whereas the equivalent ion for 2 at m/z 121 ([M-1]+) was not detected, suggesting the presence of 2d ([1H,3H]-2, MW 128), with a molecular ion at m/z 128 and a fragment ion at m/z 97. The ion intensity at m/z 97 (28–30%) observed in all the pentane extracts cannot be entirely accounted for by the low level of 2d, as estimated from the intensity at m/z 128, and therefore suggests the presence of 2c ([1H,3H]-2, MW 129) that also showed a fragment ion at m/z 97. It thus appears that the longer feeding period until stage 5 led to the incorporation of [1H,3H]-1 into 2a–2d and 3a–3d.

The accumulation of 3 may have started prior to the accumulation of 2 based on the detection of 3a–3d, but not of 2a–2d, in immature flowers at stage 2 in Experiment 1. The similar labeling patterns of 2a–2d and 3a–3d in both flower types may be ascribed to equilibrium between 2a–2d and 3a–3d through routes d and e, but do not give any direct evi-

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Table 1. Relative Ion Intensities of Ions at m/z 97, m/z 98, m/z 128, and m/z 130 of [1H,3H]-2-Phenylethanol (2a–2d) Detected as Volatile or Glucosides in Flowers of Rosa damascena Mill. and R. ‘Hoh-Jun’, and Those Predicted10 for 2a–2d

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/z 97</th>
<th>m/z 98</th>
<th>m/z 128</th>
<th>m/z 129</th>
<th>m/z 130</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. damascena volatiles</td>
<td>28.0</td>
<td>4.0</td>
<td>20.0</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>R. damascena glucoside</td>
<td>29.0</td>
<td>4.0</td>
<td>20.0</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>R. ‘Hoh-Jun’ volatiles</td>
<td>29.0</td>
<td>3.9</td>
<td>20.0</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>R. ‘Hoh-Jun’ glucoside</td>
<td>30.0</td>
<td>4.0</td>
<td>20.0</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>100</td>
<td>36.0</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>100</td>
<td>64.0</td>
<td>36.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>2c</td>
<td>100</td>
<td>64.0</td>
<td>36.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td>100</td>
<td>64.0</td>
<td>36.0</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

10) The ratio of the intensities for 2 was 100/64.0/36.0/3.0 for m/z 91, m/z 92, m/z 122, m/z 123.
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Evidence for 3b–3d being derived from 2b–2d either via [\textsuperscript{13}H\textsubscript{n}] phenylacetaldehyde or [\textsuperscript{13}H\textsubscript{n}] phenyllactic acid (routes b and c, Figs. 1 and 2) or from [\textsuperscript{13}H\textsubscript{n}] phenyllactic acid \(\beta\)-D-glucopyranoside (route f) (Fig. 1). However, 3a may have been transformed exclusively from [\textsuperscript{13}H\textsubscript{8}] phenylacetaldehyde via [\textsuperscript{13}H\textsubscript{8}] for, since no other pathway can be envisaged for this conversion. We are now focusing on the identification of hypothetical intermediates shown in Figs. 1 and 2 to clarify which of the possible biosynthetic pathways actually operate in yielding 2 and 3 in rose petals.

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