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Production of 2-Phenylethanol in Roses as the Dominant Floral Scent Compound from L-Phenylalanine by Two Key Enzymes, a PLP-Dependent Decarboxylase and a Phenylacetaldehyde Reductase

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We investigated the biosynthetic pathway for 2-phenylethanol, the dominant floral scent compound in roses, using enzyme assays. L-[$^2H_8$] Phenylalanine was converted to [$^2H_8$] phenylacetaldehyde and [$^2H_8$]-2-phenylethanol by two enzymes derived from the flower petals of *R.* 'Hoh-Jun,' these being identified as pyridoxal-5′-phosphate-dependent L-aromatic amino acid decarboxylase (AADC) and phenylacetaldehyde reductase (PAR). The activity of rose petal AADC to yield phenylacetaldehyde was nine times higher toward L-phenylalanine than toward its D-isomer, and this conversion was not inhibited by iproniazid, a specific inhibitor of monoamine oxidase. Under aerobic conditions, rose petal AADC stoichiometrically produced NH_3 together with phenylacetaldehyde during the course of decarboxylation and oxidation, followed by the hydrolysis of L-phenylalanine. Phenylacetaldehyde was subsequently converted to 2-phenylethanol by the action of PAR. PAR showed specificity toward several volatile aldehydes.

Key words: rose flower; 2-phenylethanol; aromatic L-amino acid decarboxylase; phenylacetaldehyde reductase; biosynthetic pathway

2-Phenylethanol (2PE) is an aromatic alcohol with a rose-like odor and it is emitted from several plant tissues and microorganisms. 2PE contributes to the flavor of fruits, vegetables and some foods and is also an important aroma used in fragrance products.[1)] The damask roses (*Rosa damascena*, commonly used to produce essential oil and rose water, and *R.* 'Hoh-Jun') are known to emit 2PE as a dominant aroma compound.[1,2)] Chemical and biotechnological production of 2PE have been attempted.[1,3)] Despite its simple chemical structure, with a C6+C2 skeleton, the biosynthetic pathway leading to 2PE has not been completely clarified. Bugorskii and Zaprometov[4)] have reported for the first time the biogenesis of 2PE in roses, and concluded that phenylpyruvate or phenylacetic acid were intermediates in the pathway from L-phenylalanine (L-Phe). Albertazzi *et al.*[5)] have proposed phenylpyruvate as the intermediate in the production of 2PE by yeast fermentation, using L-Phe as the sole carbon source.

We also confirmed that L-Phe is the precursor of 2PE and its β-D-glucopyranoside (2PEG) using feeding experiments of L-[$^2H_8$] Phe to intact flowers of *R. damascena* Mill. and *R.* 'Hoh-Jun'.[6)] Furthermore, we showed by feeding experiments that 2PE and 2PEG were synthesized with retention of the α-hydrogen atom of L-Phe.[7)] These results strongly suggested that the biosynthetic pathway of 2PE from L-Phe must be different from those already proposed, because the α-hydrogen atom of L-Phe must be abstracted to yield either of the intermediates, phenylpyruvate or phenylacetic acid.

We therefore proposed three plausible biosynthetic

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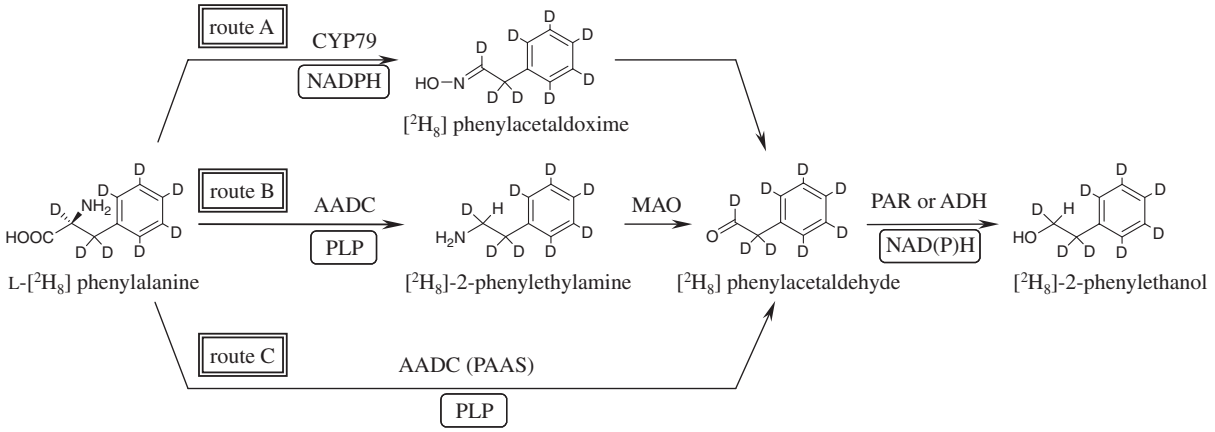
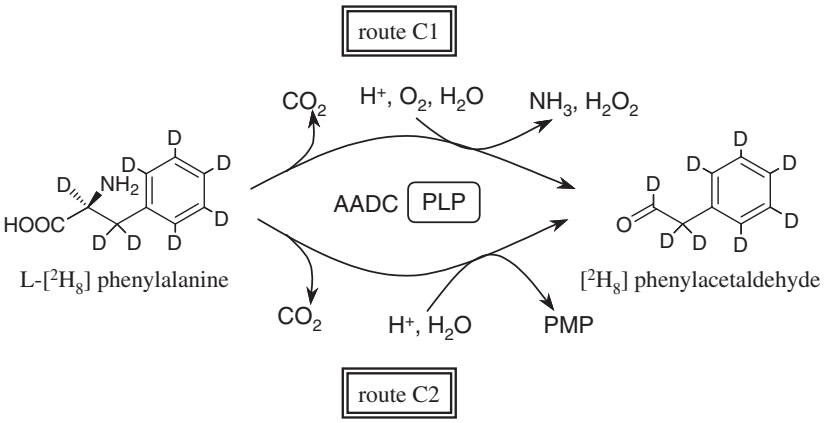
Abbreviations: 2PE, 2-phenylethanol; Phe, phenylalanine; PAld, phenylacetaldehyde; ADC, L-amino acid decarboxylase; AADC, aromatic L-amino acid decarboxylase; PAR, phenylacetaldehyde reductase; MAO, monoamine oxidase; ADH, alcohol dehydrogenase; CHAPS, 3-[(cholamidopropyl)dimethylammonio] propanesulfonic acid



Scheme 1. Hypothetical Biogenetic Pathways for the Production of [$^2H_8$] PAld and [$^2H_8$]-2PE from L-[$^2H_8$] Phe. D denotes deuterium.



Scheme 2. Hypothetical Biogenetic Pathways for the Production of [$^2H_8$] PAld from L-[$^2H_8$] Phe. D denotes deuterium.

pathways for 2PE, as shown in Scheme 1. The first possibility involves the oxidative decarboxylation of L-Phe by an enzyme of the CYP79 family[8] to produce the intermediate, phenylacetaldoxime, which is hydrolyzed to yield a phenylacetaldehyde (PAld). PAld is successively transformed to 2PE by the action of an alcohol dehydrogenase (ADH) or PAld reductase (PAR) (route A in Scheme 1). We attempted to detect the plausible intermediate, phenylacetaldoxime, in the petals of these rose flowers, but this compound could not be detected even in a trace amount. However, when [$^2H_8$] phenylacetaldoxime was fed to intact flowers, it was converted to [$^2H_8$]-2PE and [$^2H_8$]-2PEG in a high yield with retention of the α-hydrogen atom. We thus hypothesize the involvement of an enzyme from the CYP79 family.[7]

The second pathway is same as the pathway in tomato fruits (route B in Scheme 1). Tieman *et al.*[9] have recently reported a small family of aromatic L-amino acid decarboxylases (AADC) from tomatoes that catalyzes the conversion of L-Phe to 2-phenylethylamine ($2PNH_2$), which is then converted to 2PE through PAld using monoamine oxidase (MAO), and PAR. The α-hydrogen atom of L-Phe is not abstracted on this pathway.

The third pathway involves L-Phe being directly converted into PAld by AADC. Although L-amino acid decarboxylases (ADCs) in general catalyze the conversion of amino acids to the corresponding amines,[10] it has also been shown that they catalyze the conversion of amino acids to the corresponding aldehydes as minor reactions. Oxidative deamination occurs under aerobic conditions[11,12] (route C1 in Scheme 2) and half-transamination takes place under anaerobic conditions[13,14] (route C2 in Scheme 2). However, quite recently, Kaminaga *et al.*[15] have reported that a petunia AADC, designated as plant phenylacetaldehyde synthase (PAAS), directly converted L-Phe to PAld under the aerobic condition as a major reaction. PAAS produces stoichiometric amounts of PAld, CO_2, NH_3, and H_2O_2. 2PE must be produced from L-Phe with retention of its α-hydrogen atom by the action of AADC or PAAS, followed by the reduction either by PAR or ADH (route C in Scheme 1). We therefore proposed this pathway as the third possibility.

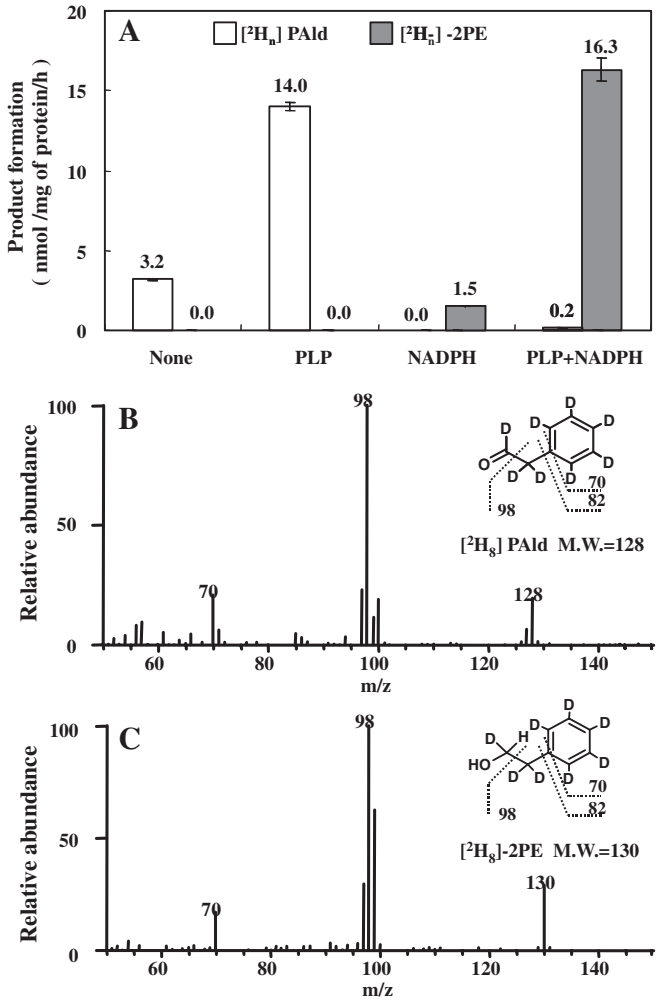


Fig. 1. Effects of PLP and NADPH on the Conversion of L-[$^2H_8$] Phe ([$^2H_8$]/[$^2H_{n\ (n=7–0)}$] $\geqq$ 83/17) into [^2H_n] PAld and [^2H_n]-2PE by Crude Enzymes Prepared from the Flower Petals of *R.* 'Hoh-Jun'.

A, product formation from L-[$^2H_8$] Phe by the crude enzymes either in the presence or absence of PLP and/or NADPH. The concentrations of PLP and NADPH were 0.05 mM and 1 mM, respectively. Data shown represent the mean value ± standard error from triplicate experiments. B, MS data for [^2H_n] PAld converted from L-[$^2H_8$] Phe, and the plausible fragmentation pattern of [$^2H_8$] PAld. C, MS data for [^2H_n]-2PE converted from L-[$^2H_8$] Phe and the plausible fragmentation pattern of [$^2H_8$]-2PE. The other reaction conditions are as described in the Experimental section.

We report here the biosynthetic pathway of 2PE from L-Phe *via* PAld that we have already proposed,[16–18] with the empirical data presented in detail using enzyme systems prepared from the flowers of *R.* 'Hoh-Jun'.

Results and Discussion

Effects of PLP and NADPH on the crude enzyme reaction

To elucidate the biosynthetic pathway of 2PE from L-Phe, the effects of the possible coenzymes, NADPH (for CYP79, and PAR or ADH activity) and PLP (for AADC activity), were examined using crude enzymes prepared from lyophilized rose petals.

The crude enzyme slightly converted L-[$^2H_8$] Phe ([$^2H_8$]/[$^2H_{n\ (n=7–0)}$] $\geqq$ 83/17; details of the isotopomer ratio will be discussed in the following section) to yield 3.2 nmol/mg of protein/h of [$^2H_{n(n=8,7,6)}$] PAld with retention of the α-deuterium atom of L-[$^2H_8$] Phe, whereas the conversion increased by 4 times (14.0 nmol/mg of protein/h) by adding PLP (Fig. 1A and B). The production of PAld in the absence of these coenzymes may have been due to the effects of remaining PLP in the crude enzyme extracts, although it was prepared without any addition of PLP to stabilize the enzymes. Thus, PLP was suggested to be essential for the conversion of L-Phe into PAld. On the other hand, in the presence of only NADPH, the crude enzyme slightly converted L-[$^2H_8$] Phe to yield 1.5 nmol/mg of protein/h of [$^2H_8$]- and [$^2H_7$]-2PE (Fig. 1A and C). These 2PEs must have been derived from [$^2H_{n\ (n=8,7,6)}$] PAld produced by the crude enzymes without the addition of any coenzymes. When both PLP and NADPH were added to the crude enzyme extracts, [$^2H_8$]- and [$^2H_7$]-2PEs were produced from L-[$^2H_8$] Phe in almost same amounts as [$^2H_{n\ (n=8,7,6)}$] PAld produced in the presence

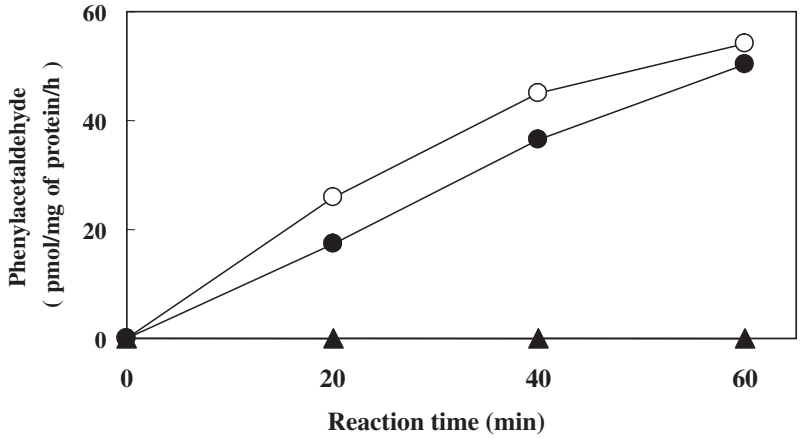


Fig. 2. Effect of a Hydroxylamine Treatment on the Conversion of L-Phe to PAld by the Crude Enzymes.

The crude enzyme extract was treated with 5 mM hydroxylamine at 25 °C for 15 min and then passed through a PD10 column to remove hydroxylamine. ○, non-treated control; ●, hydroxylamine-treated enzyme + 0.5 mM PLP; ▲, hydroxylamine-treated enzyme. The other reaction conditions are as described in the Experimental section. Data shown represent the mean value from duplicate experiments.

of only PLP. Thus, NADPH was necessary for the conversion of PAld into 2PE. Furthermore, PAld synthesis was inhibited by a hydroxylamine treatment, and restored substantially by a PLP treatment (Fig. 2). Based on the results so far obtained, PLP was necessary for PAld production from L-Phe, but NADPH was not necessary.

In the case of CYP79 catalyzing the conversion of L-Phe to phenylacetaldoxime, PAld and/or 2PE should mainly increase by adding NADPH to the reaction mixture, because we have already confirmed that phenylacetaldoxime was easily converted to PAld even by a non-enzymatic reaction in our preliminary examinations. When L-Phe was treated with the enzymes in the presence of NADPH, we didn't detect any phenylacetaldoxime in the reaction mixtures by GC-SIM analysis. Furthermore, as shown in Fig. 1A, no increment in PAld- and 2PE-production was observed even when NADPH was added to the reaction mixture, suggesting the negligible contribution of CYP79 (route A in Scheme 1).

We could confirm here the enzymatic conversion of L-Phe to 2PE *via* PAld, with retention of the α-hydrogen atom of L-Phe, as has previously been reported in intact rose flowers.[6,7] It became clear that a PLP-dependent enzyme was involved in the conversion of L-Phe into PAld (routes B and C in Scheme 1) and that an enzyme-catalyzing reduction of PAld was involved in 2PE production in the presence of NADPH.

Role of MAO in 2PE synthesis

PAld was detected in the reaction mixture when L-Phe was incubated with the crude enzymes, whereas $2PNH_2$ was not detected at all. However, when $2PNH_2$ was used as a substrate, it was converted to PAld in the reaction mixture (Table 1), suggesting the presence of a monoamine oxidase. In support of this, the conversion of $2PNH_2$ was inhibited by iproniazid, a specific inhibitor of monoamine oxidase.[19] However, iproniazid did not inhibit the production of PAld when L-Phe was used as the substrate.

Table 1. Effects of the Inhibitor of Monoamine Oxidase, Iproniazid, on the Crude Enzymatic Conversion of L-Phe and $2PNH_2$ to PAld

Data shown represent the mean value ± standard error from triplicate experiments.

Substrate and inhibitor	nmol PAld/ mg of protein/h
L-Phe (5 mM)	23.3 ± 0.1
L-Phe (5 mM) + iproniazid (2 mM)	25.0 ± 0.6
$2PNH_2$ (1 mM)	10.6 ± 0.3
$2PNH_2$ (1 mM) + iproniazid (2 mM)	1.9 ± 0.1

This apparent discrepancy can be explained by the presence of two distinct types of PAld-producing activity in the crude enzyme extract, as shown in Fig. 3. Using $2PNH_2$ as the substrate for evaluating the PAld-producing activity (MAO activity) in each fraction, this activity was detected in peak 1. When L-Phe was used as the substrate, the PAld-producing activity was detected in peak 2. Iproniazid inhibited the activity of MAO detected in peak 1, whereas the PAld-producing activity detected in peak 2 was not inhibited by iproniazid. Accordingly, peak 2 did not have MAO activity.

As already mentioned, although we detected MAO activity in the crude enzymes, PAld-producing activity was not inhibited by iproniazid, and no PNH_2 was detected in any reactions. We therefore confirmed that L-Phe was directly converted to PAld by the action of PLP-dependent AADC (route C in Scheme 1).

Partial purification and identification of by-products of the rose petal AADC reaction

Rose petal AADC was partially purified by successive chromatography on Super Q and Mono Q columns,

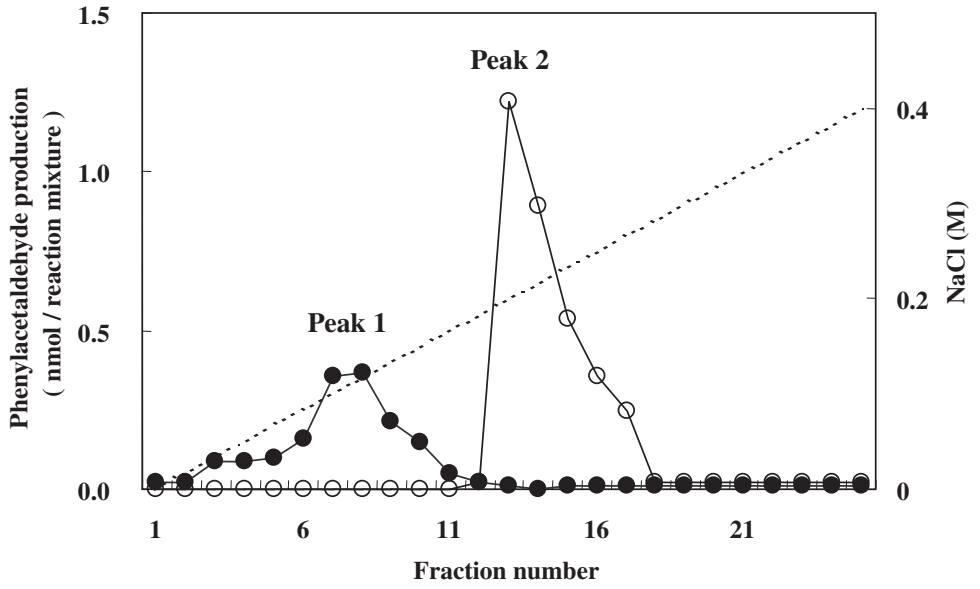


Fig. 3. Chromatographic Profiles of the PAld-Producing Activities with Two Different Substrates.
○, PAld-producing activity was assayed with L-Phe (5 mM) as a substrate; ●, PAld producing activity was assayed with $2PNH_2$ (1 mM) as a substrate; The other reaction conditions are as described in the Experimental section.

Table 2. Production of PMP, NH_3, and PAld from L-Phe in the Presence of PLP by Purified Enzymes from the Flowers of *R.* 'Hoh-Jun'

Data shown represent the mean value ± standard error from triplicate experiments.

Product	nmol/mg of protein/h
PAld	91.8 ± 4.0
NH_3	101.8 ± 5.4
PMP	0.9 ± 0.5
H_2O_2	67.3 ± 8.2

resulting in a 9-fold increase in activity when compared with the crude enzyme extract (recovery yield: 29%). The molecular weight of rose petal AADC was estimated to be 120 kDa based on chromatography on a Superdex 200 10/300 GL column.

As shown in Scheme 2, we can propose the hypothetical biosynthetic pathways for the production of PAld and 2PE from L-Phe. As an example (route C2 in Scheme 2), the Schiff base of L-Phe with PLP could be enzymatically decarboxylated, followed by hydrolysis to yield PAld and pyridoxamine 5′-monophosphate (PMP).[13,14] Alternatively (route C1 in Scheme 2), decarboxylation of the Schiff base followed by oxidation and hydrolysis would result in the production of PAld together with NH_3 and H_2O_2.[11,12,15] In this reaction, PLP is regenerated. As shown in Table 2 and Fig. 4, the molar amount of NH_3 produced was almost equal to that of PAld, while the amount of PMP was 1% of that of PAld. Although the amount of H_2O_2 was somewhat lower than the amount of PAld (Table 2), it was confirmed that H_2O_2 was produced in the reaction mixture. In addition, under semi-anaerobic conditions, the amount of PAld synthesized was only 40% of that synthesized under aerobic conditions (data not shown), indicating that PAld was synthesized from L-Phe under aerobic conditions. We also confirmed that such reaction products as PAld did not inhibit the enzyme activity (data not shown). These data strongly suggest that 2PE was synthesized from L-Phe *via* route C1 in Scheme 2, *i.e.*, the Schiff base was decarboxylated and oxidized by O_2 to release imine, followed by hydrolysis to produce PAld, NH_3 and H_2O_2. Finally, PAld thus produced was converted to 2PE by the action of reductase or dehydrogenase.

Substrate specificity of rose petal AADC

The activity of rose petal AADC toward several aromatic and aliphatic amino acids was measured by using HPLC to detect the production of NH_3, as described in the experimental section. Rose petal AADC converted L-Phe (relative activity = 100) to PAld, whereas it showed much lower activity toward D-Phe (4.8). The enzyme showed no activity toward either L-tryptophane, L-tyrosine, or L-arginine. Thus, rose petal AADC was found to be a specific enzyme for L-Phe.

Cloning of AADC and its enzyme characteristic of recombinant AADC

We have cloned the full-length *AADC* cDNA (accession no. AB305071), using the Rose EST sequence,[20] and expressed the gene in *Escherichia coli*. The protein sequence had 68% homology with *Aristolochia* tyrosine decarboxylase.[16] Although the recombinant AADC could not be completely purified, both the rose-derived and recombinant AADCs converted L-Phe to PAld and NH_3 (Fig. 5), with retention of the α-hydrogen atom. Quite recently, Kaminaga *et al.* have cloned and characterized a plant PAld synthase (PAAS) from the

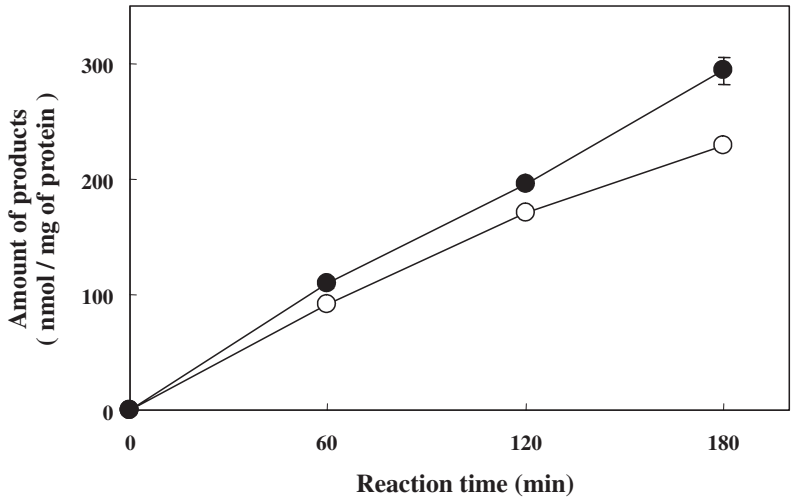


Fig. 4. Time-Course Characteristcs of PAld and NH_3 Production from L-Phe by Partially Purified Enzymes from the Flowers of *R.* 'Hoh-Jun' in the Presence of PLP.

○, PAld; ●, NH_3. The reaction conditions are as described in the Experimental section. Data shown represent the mean value ± standard error from triplicate experiments.

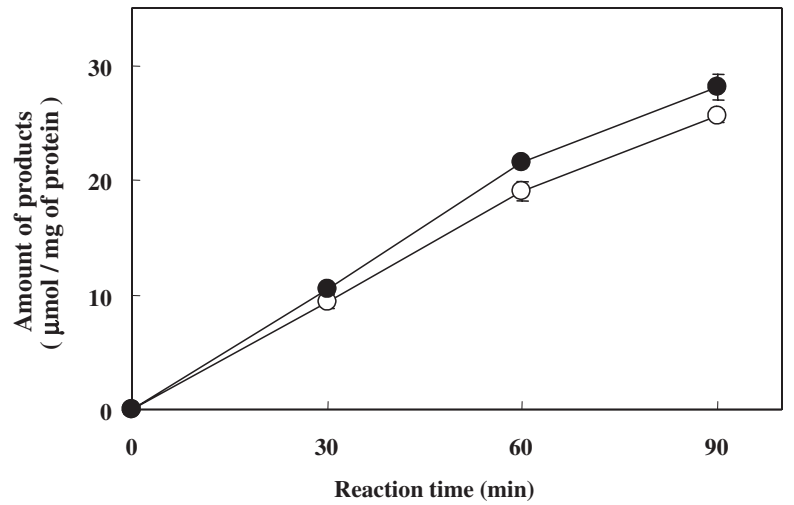


Fig. 5. Time-Course Characteristics of PAld and NH_3 Production from L-Phe by Expressed AADC in the Presence of PLP.

○, PAld; ●, NH_3. The reaction conditions are as described in the experimental section. Data shown represent the mean value ± standard error from triplicate experiments.

petunia plant as a bifunctional homotetrameric enzyme that catalyzed the decarboxylation and oxidation of L-Phe.[15] The amino acid sequence of the expressed AADC had 99% homology with this PAAS.

Partial purification and characterization of PAld reductase (PAR)

PAld reductase (PAR) was partially purified from the crude enzyme mixture by chromatography on the columns of Super Q and Mono Q. The enzyme efficiently converted PAld to 2PE in the presence of either NADPH or NADH (Fig. 6A), slightly higher PAR activity being detected when NADPH was used as a coenzyme instead of NADH. The PAR activity was 10 times higher than the ADH activity (Fig. 6B). This strongly indicates that the speed of conversion of PAld to 2PE must have been much higher than that of the formation of PAld from 2PE in the flower petals. The molecular weight of the reductase, PAR, was estimated to be 34 kDa based on Superdex 200 10/300 GL column chromatography.

As shown in Table 3, PAld (relative activity = 100) was the best substrate for PAR among the volatile aldehydes. The enzyme showed some activity toward 2-phenylpropionaldehyde (72), (*S*)-(−)-citronellal (59), hexylaldehyde (28), 3-phenylpropionaldehyde (18), (*R*)-(+)-citronellal (15), and citral (1). The other aldehydes, 4-hydroxybenzaldehyde, benzaldehyde, *trans*-cinnamaldehyde and salicylaldehyde, were not reduced to the corresponding alcohols. PAR did not reduce any keto-

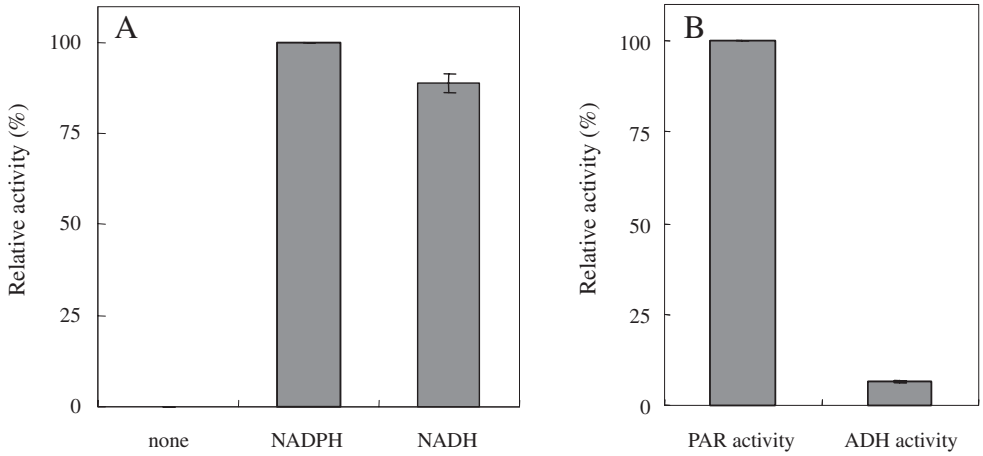


Fig. 6. Coenzyme Requirements and Reaction Direction of the PAR Fraction.

A, Coenzyme requirements. The PAR activity was assayed in the presence of 1 mM NADH or 1 mM NADPH. The activity of the NADPH sample (1.5 mmol/mg of protein/h) is regarded as 100%. B, Reaction direction of PAR. The reducing activity (PAR activity) was measured as described in the Experimental section. The oxidative activity (ADH activity) in the presence of $NADP^+$ was evaluated by a GC–MS analysis of the 2PE production. The reaction mixture contained 1 mM 2PE, 1 mM $NADP^+$, and 30 µl of the PAR fraction. The PAR activity (1.3 mmol/mg of protein/h) is regarded as 100%. All data shown represent the mean value ± standard error from triplicate experiments.

Table 3. Substrate Specificity of PAR

Relative activity shows the percentage activity of the detected using phenylacetaldehyde. Data shown represent the mean value ± standard error from triplicate experiments.

Compound	Relative activity (%)
phenylacetaldehyde	100.0 ± 2.3
2-phenylpropionaldehyde	71.7 ± 3.5
(*S*)-(−)-citronellal	58.8 ± 0.7
hexylaldehyde	28.4 ± 1.1
3-phenylpropionaldehyde	18.2 ± 3.0
(*R*)-(+)-citronellal	15.0 ± 1.2
citral	1.0 ± 1.9
4-hydroxybenzaldehyde	0
trans-cinnamaldehyde	0
benzaldehyde	0
salicylaldehyde	0
acetophenone	0
2-butanone	0
methyl butylketone	0
methyl pentylketone	0

carbonyl groups of acetophenone, methyl butylketone, methyl pentylketone, or 2-butanone (Table 3).

In the case of enantiomers of citronellal, a higher amount of (*S*)-citronellal was reduced than its (*R*)-isomer, indicating that the PAR activity was affected by the chirality of the C-6 position. This may also be related to the differences in the enzyme's specificity toward several other aldehydes with aromatic groups such as benzaldehyde, 4-hydroxybenzaldehyde, phenyl propionaldehyde, *trans*-cinnamaldehyde, and PAld. Our data strongly suggest that the PAR activity must have been affected by the stereostructures of the substrates used. It is therefore quite important to clarify the protein sequence of PAR, and to express the gene, in order to analyze the 3D structure of its substrate-binding pocket.

Formation of [^2H_n] PAld and [^2H_n]-2PE from L-[$^2H_8$] Phe in the presence of AADC and/or PAR

When L-[$^2H_8$] Phe ([$^2H_8$]/[$^2H_{n\ (n=7–0)}$] ≧ 83/17) was incubated with AADC in the absence of PAR, the enzyme produced 70% of [1,2,2,2′,3′,4′,5′,6′-$^2H_8$] PAld, 28% of [1,2,2′,3′,4′,5′,6′-] and/or [2,2,2′,3′,4′,5′,6′-$^2H_7$] PAld, and 2% of [$^2H_6$] PAld isotopomers over a period of 60 min at pH 7.0 (Fig. 7A). Under the same reaction conditions, but in the presence of both the enzymes, AADC and PAR, L-[$^2H_8$] Phe was converted to [1,2,2,2′,3′,4′,5′,6′-$^2H_8$]-/([1,2,2′,3′,4′,5′,6′-$^2H_7$]- + [2,2,2′,3′,4′,5′,6′-$^2H_7$])-2PE in the ratio of 87/13 (Fig. 7B).

To clarify the difference of isotopomer ratio between [^2H_n] PAld and [^2H_n]-2PE, we examined the stability of [$^2H_8$] PAld (the ratio of [$^2H_8$]/[$^2H_7$]/[$^2H_6$] = 88/10/2, Fig. 8A) in various pH solutions. When [$^2H_8$] PAld was left in the reaction buffer (0.1 M potassium phosphate, pH 6.0) for 60 min in the absence of enzymes, there was a decline in the ratio of the ^2H atoms of [$^2H_8$] PAld ([$^2H_8$]/[$^2H_7$]/[$^2H_6$] = 80/18/2 (Fig. 8B). Further decline was observed at pH 7.0, the ratio for [$^2H_8$]/[$^2H_7$]/[$^2H_6$]PAld = 65/32/3 (Fig. 8C) being observed, this being similar to the ratio for ([$^2H_8$]/[$^2H_7$]/[$^2H_6$] PAld = 69/27/4) observed in the enzymatic reaction mixture (Fig. 7A). When [^2H_n] PAld was incubated in the buffer at pH 8.0, an exchange of ^2H atoms with a proton at the 2 position occurred with a higher ratio (Fig. 8D). We therefore confirmed an exchange of the ^2H atoms of [1,2,2,2′,3′,4′,5′,6′-$^2H_8$] PAld with protons to yield [1,2,2′,3′,4′,5′,6′-$^2H_7$] and [1,2′,3′,4′,5′,6′-$^2H_6$] PAld by keto-enol equilibrium at the higher pH value. On the other hand, as shown in Fig. 7B, L-[^2H_n] Phe ([$^2H_8$]/[$^2H_{n\ (n=7–0)}$] ≧ 83/17) was converted to [^2H_n]-2PE ([$^2H_8$]-/[$^2H_7$]-2PE = 87/13) without loss of ^2H atoms in the presence of AADC and PAR, even under pH 7.0. We therefore concluded that [$^2H_8$] PAld

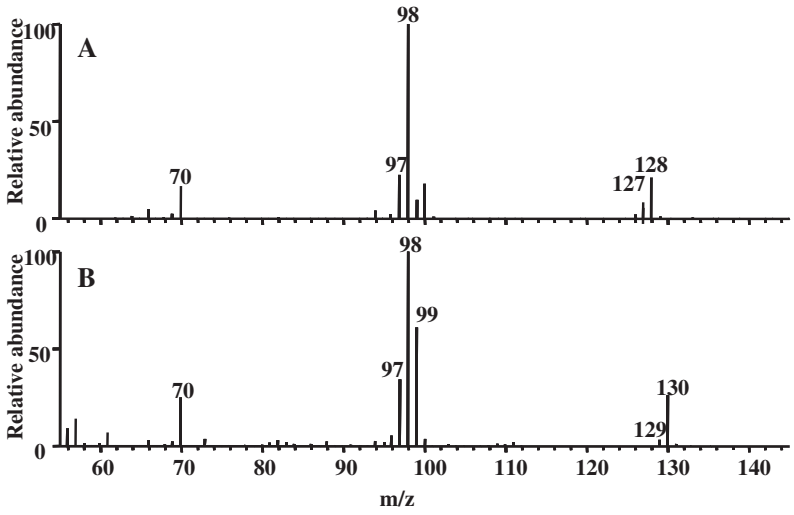


Fig. 7. GC–MS Profiles of the Products Resulting from the Conversion of L-[$^2H_8$] Phe ([$^2H_8$]/[$^2H_{n\ (n=7–0)}$] $\geqq$ 83/17) into [^2H_n] PAld and [^2H_n]-2PE by the Partially Purified Enzymes.

A, [^2H_n] PAld, product of the AADC reaction. B, [^2H_n]-2PE, produced by rose petal AADC and PAR combined. The pH value of the reaction mixture was 7.0 and the reaction time was 60 min.

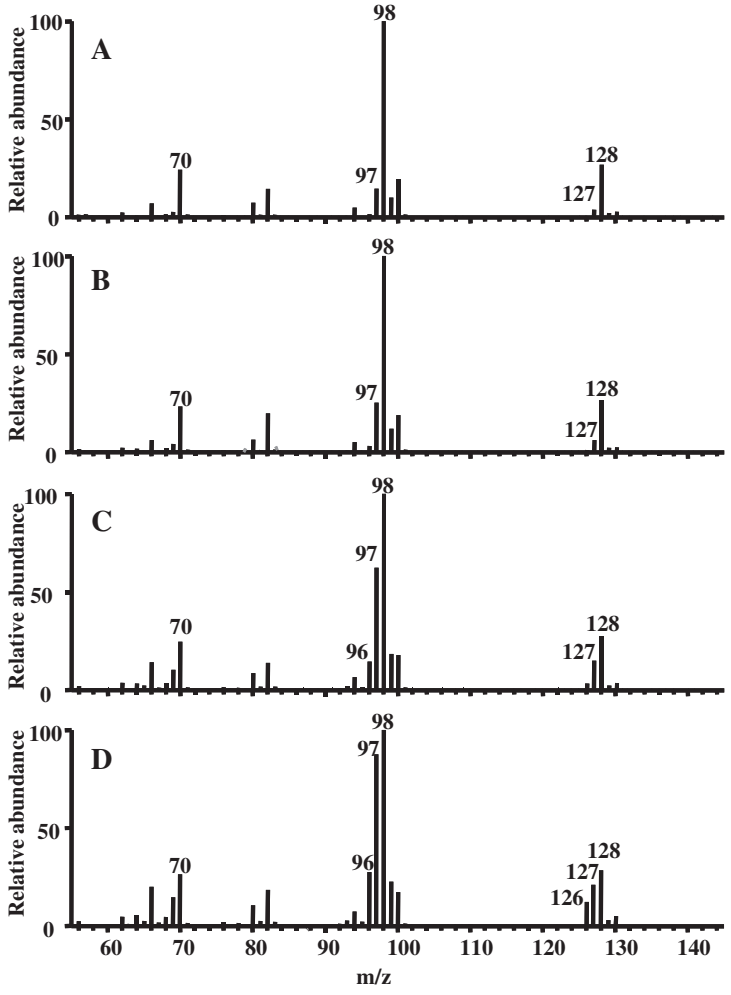


Fig. 8. Stability of [$^2H_8$] PAld ([$^2H_8$-]/[$^2H_7$]/[$^2H_6$] = 88/10/2) in Various pH Solutions.

A, [$^2H_8$] PAld ([$^2H_8$]/[$^2H_7$]/[$^2H_6$] = 88/10/2. B, pH 6.0. C, pH 7.0. D, pH 8.0. [$^2H_8$] PAld ([$^2H_8$]/[$^2H_7$]/[$^2H_6$] = 88/10/2, 1 mM) was left in a 10 mM phosphate buffer at pH 6.0 (B), pH 7.0 (C), and pH 8.0 (D) for 60 min. Then [^2H_n] PAld was extracted with a mixture of EtOAc and hexane (1:1 v/v), and subjected to GC–MS analyses.

produced from L-[$^2H_8$] Phe by AADC was immediately converted into [$^2H_8$]-2PE by PAR in the reaction mixture.

Conclusion

We partially purified for the first time the important rose petal enzymes, AADC and PAR, which are involved in the biosynthesis of 2PE in the flowers of such roses as *R.* 'Hoh-Jun' and *R. damascena* Mill. We could detect MAO in the crude enzymes prepared from rose petals, but this enzyme was not involved in the synthesis of PAld from L-Phe. Under aerobic conditions, rose petal AADC produced PAld, NH_3, and H_2O_2 without the production of any $2PNH_2$.

We have cloned the full-length *AADC* cDNA, using the Rose EST sequence,[20] and expressed the gene in *Escherichia coli*. Both the rose-derived and expressed AADCs functioned similarly to convert L-Phe to PAld and NH_3, with retention of the α-hydrogen atom.[16–18] Therefore, rose AADC was an enzyme with the same function as PAAS.

We also successfully partially purified the enzyme, PAR, which is involved in the transformation of PAld to 2PE, from the flowers of *R.* 'Hoh-Jun.' This enzyme activity was affected by the chirality and stereochemistry of the substrates. It is therefore quite important to clarify the protein sequence of PAR, and to express the gene, in order to analyze the 3D structure of its substrate-binding pocket.

Experimental

Chemicals and biochemicals. L-[2,3,3,2′,3′,4′,5′,6′-$^2H_8$] Phe (L-[$^2H_8$] Phe, 98 atom % ^2H, Aldrich) was used. For the ^{1}H-NMR spectral analyses of the methyl ester of L-[$^2H_8$] Phe, the $^2H/H$ ratio was evaluated to be 83/17, 97/3, and 96/4 for H-2, 2 × H-3, and H-2′-6′, respectively. Therefore, the isotopomer ratio of this reagent was estimated to be [$^2H_8$]/[$^2H_{n\ (n=7-0)}$] $\geqq$ 83/17. All chemicals were of the highest grade commercially available, unless noted otherwise.

Synthesis of [1,2,2,2′,3′,4′,5′,6′-$^2H_8$] phenylacetaldehyde ([1,2,2,2′,3′,4′,5′,6′-$^2H_8$] PAld). L-[$^2H_8$] Phe (50 mg, 0.29 mmol) and pyridoxal 5′-phosphate (80 mg, 0.30 mmol) were added to a mixture of 2.7 µl $C^2H_3O^2H$ and 0.5 µl ^2H_2O in a sealed vial, and then heated to 75 °C under pressure (2,000 hPa) for 2 h. The reaction vial was allowed to cool, and then 5 ml of ^2H_2O was added. [$^2H_8$] PAld was extracted with a mixture of hexane-ethylacetate (1:1 v/v, 5 ml, 3 times each), and removal of the solvent *in vacuo* at 30 °C yielded [$^2H_8$] PAld (4.90 mg, 13.2% yield). The chemical structure and its molecular weight were confirmed by GC–MS that showed ions at m/z 128 (16%, M^+ for [$^2H_8$] PAld), m/z 98 (100%, $M^+ - C^2HO$), and m/z 82 (5%, $M^+ - C^2H_2C^2HO$). The ratio of the deuterium labels was determined by ^{1}H-NMR spectral analyses of the 2,4-dinitro-phenylhydrazone of [$^2H_8$] PAld, which was prepared by the usual manner in $C^2H_3O^2H$ in the presence of ^{2}HCl. The $^2H/H$ ratio was evaluated to be 91/9, 86/14, and 97/3 for H-1, 2 × H-2, and H-2′-6′, respectively, by comparing the ^{1}H-NMR signal intensities for H-1, 2 × H-2, and H-2′-6′, with those for H-3, 5″ and 6″ of the 2,4-dinitro-phenyl group. The isotopomer ratio was calculated to be 88/10/2 for [1,2,2,2′,3′,4′,5′,6′-$^2H_8$] PAld/[2,2,2′,3′,4′,5′,6′-$^2H_7$] PAld/[$^2H_6$] PAld based on the GC–MS chromatographic analyses (*vide infra*).

Synthesis of pyridoxamine 5′-phosphate (PMP). PMP was synthesized as described by Yang *et al.*[21] Pyridoxamine (50 mg, 0.30 mmol) and H_3PO_4 (500 mg, 5 mmol) were heated at 100 °C for 8 h in a sealed vial, and then left to cool. EtOH (5 ml) was added, and the resulting precipitate was obtained by filtration and successively washed with EtOH and Et_2O. The precipitate was dissolved in a minimum amount of water and then subjected to preparative cellulose TLC (Merck, Art. 5577, solvent; isopropanol–NH_4OH, 6:1 v/v). The PMP zone (R_f 0.08–0.12) was scraped off and eluted with MeOH. After evaporating the solvent, PMP (28 mg, 37% yield) was obtained. The LC–MS (ESI, positive) spectrum of PMP gave an ion at m/z 249 $[MH]^+$. ^{1}H-NMR (δ, 270 MHz, ^2H_2O): 7.68 (1H, H-6), 4.95 (2H, 5′-H, J_{HCOP} = 7 Hz), 4.27 (2H, H-4′), 2.41 (3H, H-2′); ^{13}C-NMR (δ, 67.5 MHz, ^2H_2O): 165.7 (C-3), 147.6 (C-2), 136.6 (C-5, d, J_{CCOP} = 7.2 Hz), 135.8 (C-4), 126.0 (C-6), 64.9 (C-5′, d, J_{COP} = 5.2 Hz), 39.3 (C-4′), 17.7 (C-2′).

^{1}H- and ^{13}C-NMR. ^{1}H- and ^{13}C-NMR analyses were conducted with either a JNM λ500A spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C, JEOL Co.) or a JNM 270 spectrometer (270 MHz for ^{1}H, 67.5 MHz for ^{13}C, JEOL Co.).

GC–MS analyses. The GC–MS analysis was conducted with a Trace DSQ system (Thermo Fisher Scientific) equipped with a Trace GC ultra (Thermo Fisher Scientific). The analytical column was an Rtx-5MS (30 m, 0.25 mm I.D., 0.25 µm D.F., Restek) and the column temperature was elevated from 60 °C (5 min hold) to 170 °C (20 °C/min) to 290 °C (40 °C/min, 3 min hold). The injector temperature was 200 °C, the ionizing voltage was 70 eV, and the scanning speed was 0.5 scan/sec with a range of m/z 40–250. Samples (1 µl) were injected by a CombiPAL autosampler (CTC Analytics). The identification of PAld, 2PE, [^2H_n] PAld and [^2H_n]-2PE were established by comparing their MS spectra with these of authentic samples and newly synthesized compounds (mentioned later). The ratio of [^2H_n]-2PE was determined from the ion intensities of the MS chromatograms at m/z 130 [M^+] for [$^2H_8$]-2PE, m/z 129 [M^+] for [$^2H_7$]-2PE, and m/z 128 [M^+] for [$^2H_6$]-2PE. The retention times of [$^2H_8$]-, [$^2H_7$]-, and [$^2H_6$]-2PE were 5.96, 5.97, and 5.98 min, respectively, whereas 2PE was detected at 6.01 min. The ratio of [^2H_n] PAld

was determined from the MS chromatograms at m/z 128 [M^+] for [$^2H_8$] PAld, m/z 127 [M^+] for [$^2H_7$] PAld, and m/z 126 [M^+] for [$^2H_6$] PAld. The retention times of [$^2H_8$], [$^2H_7$], [$^2H_6$] PAld and PAld were 5.35, 5.36, 5.37, and 5.39 min, respectively.

To confirm the presence of each isotopomer of [^2H_n] PAld and PAld, besides their molecular ions, the relative ion intensities of fragments were examined based on the theoretical fragmentation pattern and the MS data for each isotopomer; namely, m/z 100 $[M - C^2HO + {}^2H]^+$ for [$^2H_8$] PAld, m/z 99 $[M - C^2HO + H]^+$ for [$^2H_8$] PAld, m/z 98 $[M - C^2HO]^+$ for [$^2H_8$] PAld or $[M - CHO + H]^+$ for [$^2H_7$] PAld, m/z 97 $[M - CHO]^+$ for [$^2H_7$] PAld or $[M - CHO + H]^+$ for [$^2H_6$] PAld, m/z 96 $[M - CHO]^+$ for [$^2H_6$] PAld, m/z 92 $[M - CHO + H]^+$ for PAld, and m/z 91 $[M - CHO]^+$ for PAld. The presence of each isotopomer of [^2H_n]-2PE and 2PE was also confirmed based on the relative ion intensities at m/z 98 $[M - C^2H_2O]^+$ for [$^2H_8$]-2PE, m/z 97 $[C - {}^2H_2O]^+$ for [$^2H_7$]-2PE, and m/z 91 $[M - CH_2O]^+$ for 2PE.

Plant materials. Rosa 'Hoh-Jun' plants were grown on the University Farm, Faculty of Agriculture, Shizuoka University, Japan as described previously.[7] The stages of floral growth were explained previously.[7] Flowers at stages 4 (outer petal whorl opened, inner petal whorl beginning to loosen) to 6 (inner and outer whorls open, reproductive organs not visible) were harvested during April to June 2004–2006.

Preparation of crude enzymes from the flowers of R. 'Hoh-Jun'. Flowers at stages 4 to 6 were combined and crushed in liquid N_2, and then lyophilized to obtain powdered material. This material (500 mg) was homogenized in 50 ml of buffer A (0.1 M potassium phosphate, pH 7.5, containing 0.5% 3-[(cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPS, Wako Pure Chemicals), 0.25 mM PLP, 1 mM EDTA, 2 mM DTT, 0.5 mM 4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride (AEBSF, Wako Pure Chemicals), and 5% glycerol) in the presence of 3.8 g of PVPP (Polyclar 10, ISP Japan). After centrifugation (3700 × g, 20 min), the supernatant was set aside. The precipitate was resuspended in 10 ml of buffer A, and following a second centrifugation (3700 × g, 20 min), the supernatants were combined. The combined supernatant was fractionated with ammonium sulfate, and proteins that precipitated between 20% and 50% saturated ammonium sulfate were redissolved in 5 ml of buffer B (0.01 M potassium phosphate, pH 7.5, containing 0.05% CHAPS, 1 mM EDTA, 1 mM DTT, and 5% glycerol). The fraction (2.5 ml) was passed through a PD10 column (GE Healthcare) that had been equilibrated with buffer B to remove residual ammonium sulfate. The eluate was concentrated with an ultra-filtration system (Microsep MWCO 30K, Pall Life Sciences) to give the crude enzymes.

Partial purification of rose petal AADC and PAR from R. 'Hoh-Jun' and determination of their molecular sizes. The crude enzymes were further purified by chromatography using a Super Q Toyopearl column (5 ml, Tosoh) and a Mono Q 4.6/100 PE column (GE Healthcare), resulting in the partially purified enzymes, AADC and PAR. The crude enzyme solution was applied to a Super Q column equilibrated with buffer B. AADC, PAR, and MAO were eluted with a linear 0–0.5 M NaCl gradient in the same buffer. The fractions with AADC and PAR activity were collected and separated from the fraction with MAO activity. This enzyme solution was concentrated by ultrafiltration (Macrosep MWCO 30K, Pall Life Sciences) applied to a PD10 column (GE Healthcare), and then the enzyme was eluted with buffer B. This desalted enzyme solution was applied to a Mono-Q 4.6/100 PE column equilibrated with buffer B. AADC and PAR were eluted with a linear 0–0.4 M NaCl gradient in the same buffer. The fractions with AADC and PAR activity were separately collected. To determine the molecular sizes of AADC and PAR, the purified sample (100 µl) was subjected to column chromatography on a Superdex 200 10/300 GL column (0.01 M potassium phosphate, pH 7.0, containing 1 mM EDTA, 1 mM DTT, 0.05% CHAPS, and 5% glycerol) at a flow rate of 0.5 ml/min. A gel filtration standard (Bio-Rad) was used as a molecular size marker. The protein concentration was determined by the Bradford method, using bovine serum albumin as a standard.

Enzymatic reactions and detection of PAld and/or 2PE. The reaction mixtures containing 5 mM L-Phe or the L-[$^2H_8$] Phe ([$^2H_8$]/[$^2H_{n\ (n=7–0)}$] $\geqq$ 83/17) solution, 0.05 mM PLP and/or 1 mM NADPH, and 60 µl of the enzyme solution in a 0.1 M potassium phosphate buffer (pH 7.0) with a total volume of 200 µl were incubated at 35 °C for 1 h. A benzaldehyde solution (50 µg in 5 µl) was added as an internal standard to the reaction mixture. PAld, [^2H_n] PAld, 2PE, and/or [^2H_n]-2PE were extracted (3 times) with a mixture of EtOAc and hexane (1:1 v/v), and then the organic phases were combined, dried over anhydrous Na_2SO_4, and analyzed by GC–MS. The GC–MS conditions were as already described.

Detection of $2PNH_2$ and NH_3 in the enzymatic reaction mixture. In order to measure the $2PNH_2$ and NH_3 production, 5 µl of HCl (1 M), 40 µl of acetone, and 50 µl of a taurine solution (0.5 µmole/ml) were added to the reaction mixture. The solution was subjected to centrifugation (1000 × g, 3 min), and an aliquot of the supernatant (50 µl) was added to 200 µl of a 0.1 M borate buffer (pH 8.5) and 100 µl of 0.1% 9-fluorenylmethyl chloroformate (FMOC, Wako Pure Chemicals) in acetone and then incubated for 10 min at 40 °C. After filtration through a membrane (0.45 µm), the filtrate was assayed by HPLC under the following conditions: a Waters 2695 Separations Module equipped with a Waters 2475 Multi λ Fluorescent Detector and a

Cosmosil PAQ column (4.6 × 150 mm, Nacalai Tesque) were used. The solvents were A: 0.1 M NaOAc containing 5.6 mM triethylamine (pH 5.8), and B: MeCN. The column was developed by increasing the latter from 30% to 35% in 30 min, then to 90% in 35 min at a flow rate of 0.8 ml/min at 40 °C. The excitation and emission wavelengths were 315 and 270 nm, respectively. To analyze the effects of the coenzymes, PLP and/or NADPH were added in various concentrations, as shown in the legend to figures. Iproniazid (Sigma-Aldrich) was used as an inhibitor of MAO.

Detection of H_2O_2 in the enzymatic reaction mixture. The partially purified rose petal AADC fractions were replaced with a 0.01 M potassium phosphate buffer (pH 7.0). The enzyme reaction was conducted by the method just mentioned. The production of H_2O_2 in the reaction mixture was assayed with FOX reagent as described by Wolff.[22)]

Expression of rose petal AADC in Escherichia coli. The cDNA for rose petal *AADC* was cloned into the pET28a expression vector (Novagen) to generate a construct coding for a protein with an N-terminal His tag. The rose petal *AADC* cDNA was amplified from a rose EST clone[20)] and cloned into pET28a to code for a protein with a *C*-terminal His tag. The construct was verified by DNA sequencing. Recombinant AADC expressed in *E. coli* was purified using a His-Tag column (1 × 2 ml, GE Healthcare). The protein concentration was determined by the Bradford method, using bovine serum albumin as a standard.

Substrate specificity of AADC and PAR. In assays to determine the substrate specificity of AADC, the enzyme activity was assayed by measuring the NH_3 production in the reaction mixture after 60 min, as already described. The substrates used were L- and D-Phe, L-tyrosine, L-arginine, L-tryptophane, and L-valine.

PAR activity was assayed by estimating the amount of NAD^+ in the reaction mixture after 30 min. The reaction mixture contained 40 µl of an enzyme solution with 20 µl of substrate (10 mM each), 20 µl of NADH (10 mM) and 120 µl of 0.1 M potassium phosphate buffer (pH 7.0). At the end of the reaction, 300 µl of MeCN and 50 µl of hippuric acid (25 mg in 100 ml of the phosphate buffer) were added. After filtration through a membrane (0.45 µm), 10 µl of the sample solution was assayed by HPLC under the following conditions: A Waters 2695 Separations Module equipped with a Waters 2996 Photodiode Array Detector and a Mightysil RP-18 GP Aqua column (4.6 × 150 mm, Kanto Chemical) were used. The solvents were A: 50 mM phosphate buffer (pH 6.0) and B: MeCN. The column was developed by increasing the latter from 2% to 15% in 5 min, and then to 60% in 9 min at a flow rate of 1 ml/min at 35 °C. The wavelengths were 258 and 230 nm for NAD^+ and hipurric acid, respectively. The substrates used were PAld, acetophenone, 2-phenylpropionaldehyde, 3-phenylpropionaldehyde, salicylaldehyde, 4-hydroxybenzaldehyde, citral, hexylaldehyde, benzaldehyde, trans-cinnamaldehyde, (*R*)-(+)-citronellal, (*S*)-(−)-citronellal, 2-butanone, methyl butylketone and methyl pentylketone.

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References

1) Clark, G. S., Phenetyl alcohol. *Perf. Flav.*, **15**, 37–44 (1990).
2) Rusanov, K., Kovacheva, N., Vosman, B., Zhang, L., Rajapakse, S., Atanassov, A., and Atanassov, I., Microsatellite analysis of *Rosa damascena* Mill. accessions reveals genetic similarity between genotypes used for rose oil production and old Damask rose varieties. *TAG Theor. Appl. Genet.*, **111**, 804–809 (2005).
3) Etschmann, M. M. W., Bluemke, W., Sell, D., and Schrader, J., Biotechnological production of 2-phenylethanol. *Appl. Microbiol. Biotechnol.*, **59**, 1–8 (2002).
4) Bugorskii, P. S., and Zaprometov, M. N., Biosynthesis of beta-phenylethanol in rose petals. *Biokhimiia*, **43**, 2038–2042 (1978).
5) Albertazzi, E., Cardillo, R., Servi, S., and Zucchi, G., Biogeneration of 2-phenylethanol and 2-phenylethylacetate important aroma components. *Biotech. Lett.*, **16**, 491–496 (1994).
6) Watanabe, S., Hayashi, K., Yagi, K., Asai, T., MacTavish, H., Picone, J., Turnbull, C., and Watanabe, N., Biogenesis of 2-phenylethanol in rose flowers: incorporation of [$^2H_8$] L-phenylalanine into 2-phenylethanol and its β-D-glucopyranoside during the flower opening of *Rosa* 'Hoh-Jun' and *Rosa damascena* Mill. *Biosci. Biotechnol. Biochem.*, **66**, 943–947 (2002).
7) Hayashi, S., Yagi, K., Ishikawa, T., Kawasaki, M., Asai, T., Picone, J., Turnbull, C., Hiratake, J., Sakata, K., Takada, M., Ogawa, K., and Watanabe, N., Emission of 2-phenylethanol from its β-D-glucopyranoside and the biogenesis of these compounds from [$^2H_8$] L-phenylalanine in rose flowers. *Tetrahedron*, **60**, 7005–7013 (2004).
8) Wittstock, U., and Halkier, B. A., Cytochrome P450 CYP79A2 from *Arabidopsis thaliana* L. catalyzes the conversion of L-phenylalanine to phenylacetaldoxime in the biosynthesis of benzylglucosinolate. *J. Biol. Chem.*, **275**, 14659–14666 (2000).
9) Tieman, D., Taylor, M., Schauer, N., Fernie, A. R., Hanson, A. D., and Klee, H. J., Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde.

PNAS, **103**, 8287–8292 (2006).

10) Eliot, A. C., and Kirsch, J. F., Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annu. Rev. Biochem.*, **73**, 383–415 (2004).
11) Bertoldi, M., Moore, P. S., Maras, B., Dominici, P., and Voltattorni, C. B., Mechanism-based inactivation of dopa decarboxylase by serotonin. *J. Biol. Chem.*, **271**, 23954–23959 (1996).
12) Sakai, K., Miyasako, Y., Nagatomo, H., Watanabe, H., Wakayama, M., and Moriguchi, M., L-Ornithine decarboxylase from *Hafnia alvei* has a novel L-ornithine oxidase activity. *J. Biochem.*, **122**, 961–968 (1997).
13) Bertoldi, M., and Voltattorni, C. B., Reaction of dopa decarboxylase with L-aromatic amino acids under aerobic and anaerobic conditions. *Biochem. J.*, **352**, 533–538 (2000).
14) Bertoldi, M., and Voltattorni, C. B., Dopa decarboxylase exhibits low pH half-transaminase and high pH oxidative deaminase activities toward serotonin (5-hydroxytryptamine). *Protein Sci.*, **10**, 1178–1186 (2001).
15) Kaminaga, Y., Schnepp, J., Peel, G., Kish, C. M., Ben-Nissan, G., Weiss, D., Orlova, I., Lavie, O., Rhodes, D., Wood, K., Porterfield, D. M., Cooper, A. J. L., Schloss, J. V., Pichersky, E., Vainstein, A., and Dudareva, N., Plant phenylacetaldehyde synthase is a bifunctional homotetrameric enzyme that catalyzes phenylalanine decarboxylation and oxidation. *J. Biol. Chem.*, **281**, 23357–23366 (2006).
16) Sakai, M., Hirata, H., Itano, H., Sayama, H., Dohra, H., Hara, M., and Watanabe, N., Biosynthesis of 2-phenylethanol, a dominant scent compound emitted from rose flowers. Abstract from TEAC 2005, Fukui, November, 2005, pp. 433–435.
17) Watanabe, N., Hara, M., Dohra, H., Sakai, M., and Hirata, H., Japan Kokai Tokkyo Koho, 2007-189943 (August 2, 2007).
18) Watanabe, N., Mystery of rose fragrance: biosynthesis in rose. *Gendai Kagaku* (*Chemistry Today*, in Japanese), **420**, 47–54 (2006).
19) Smith, T. E., Weissbach, H., and Udenfriend, S., Studies on monoamine oxidase: the mechanism of inhibition of monoamine oxidase by iproniazid. *Biochemistry*, **2**, 746–751 (1963).
20) Guterman, I., Shalit, M., Menda, N., Piestun, D., Dafny-Yelin, M., Shalev, G., Bar, E., Davydov, O., Ovadis, M., Emanuel, M., Wang, J., Adam, Z., Pichersky, E., Lewinsohn, E., Zamir, D., Vainstein, A., and Weiss, D., Rose scent: genomics approach to discovering novel floral fragrance–related genes. *Plant Cell*, **14**, 2325–2338 (2002).
21) Yang, D., Shih, Y., and Liu, H., Chemical synthesis of stereospecifically labeled pyridoxamine 5′-phosphate. *J. Org. Chem.*, **56**, 2940–2946 (1991).
22) Wolff, S. P., Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.*, **233**, 182–189 (1994).