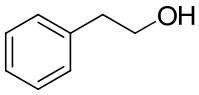
Graphical abstract

A protoplast-based approach to elucidate the biosynthesis of 2-phenylethanol in rose flowers

Ziyin Yang, Miwa Sakai, Hironori Sayama, Taku Shimeno, Koji Yamaguchi, Naoharu Watanabe *

2-phenylethanol is a dominant volatile compound in rose flowers such as *Rosa* 'Yves Piaget'. Tracer experiments in a protoplast-based system with stable isotope labeling precursors proposed the hypothetical biochemical pathway of 2-phenylethanol from shikimic acid *via* chorismic acid, L- phenylalanine, and phenylacetaldehyde.



2-Phenylethanol

Title: A protoplast-based approach to elucidate the biosynthesis of 2-phenylethanol in rose flowers

Authors: Ziyin Yang [a, b], Miwa Sakai [a], Hironori Sayama [b], Taku Shimeno [b], Koji Yamaguchi [b], Naoharu Watanabe [a, b,*]

***Affiliation*:**

[a] *Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

[b] *Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

[*] Corresponding author. Tel: +8154-2384870; fax: +8154-2384870. *Email address:* acnwata@agr.shizuoka.ac.jp (N. Watanabe).

Abstract

A protoplast-based system was used to investigate the metabolic pathway in rose flower leading from shikimic acid or L-phenylalanine (L-Phe) to 2-phenylethanol (2PE), a dominant volatile compound in rose flowers such as *Rosa damascena* 'Mill', *Rosa* 'Hoh-Jun', and *Rosa* 'Yves Piaget'. Deuterium-labeled L-Phe ([$^{2}H_{8}$]-L-Phe) was supplied to protoplasts isolated from *Rosa* 'Yves Piaget' petals. The volatile end products ([$^{2}H_{n}$]-2PE, n=6-8) and their related intermediates ([$^{2}H_{n}$]-phenylacetaldehyde, n=6-8) were detected in the protoplast-based system by GC-MS. In addition, we chemically synthesized [2,3,4,5,6-$^{13}C_{5}$] shikimic acid, a new stable isotopomer, to investigate the formation of 2PE from shikimic acid by GC-MS and NMR. It proposed the hypothetical biochemical pathway of 2PE from shikimic acid *via* chorismic acid, L-Phe, and phenylacetaldehyde. This protoplast-based system facilitates finding of metabolic intermediates and simplifies the complex branching biosynthetic pathways of floral scent to distinct individual event.

Keywords: *Rosa* 'Yves Piaget'; Rose flower; Biosynthesis; 2-Phenylethaol; Protoplast; Phenylacetaldehyde; L-Phenylalanine; Chorismic acid; [2,3,4,5,6-$^{13}C_{5}$] Shikimic acid

Introduction

Rose floral scents have been studied extensively because of commercial interest from the perfume industry and their importance in attracting pollinators. In the scent of roses, more than 400 volatiles have been identified (**Baldermann et al., 2008**). Most rose floral volatiles are classified as volatiles derived from the three major pathways, i.e. geranyl pyrophosphate (GPP) / geranylgeranyl pyrophosphate (GGPP), shikimate, and fatty acid (**Baldermann et al., 2008**). Terpenoids, i.e. monoterpenes, hemiterpenes, sesquiterpenes, and diterpenes, are formed by GPP / GGPP pathways, whereas most phenylpropanoids and benzenoids derive from shikimic acid (**Pichersky et al., 2006**). The shikimate pathway links carbohydrate metabolism to the synthesis of aromatic compounds, particularly the aromatic amino acids like L-phenylalanine (L-Phe), which can in turn act as precursors for various primary and secondary metabolites (**Dewich, 2002**).

In rose flowers such as *Rosa damascena* 'Mill', *Rosa* 'Hoh-Jun', and *Rosa* 'Yves Piaget', the most dominant volatile compound derived from the shikimate pathway is 2-phenylethanol (2PE), which is an aromatic alcohol having rose-like odour (**Baldermann et al., 2008**). In previous studies, we have confirmed that L-Phe and phenylacetaldehyde (PAld) are precursor and intermediate compound of biosynthesis of 2PE in roses respectively by supplying the stable isotope-labeled precursor to native rose flowers (**Watanabe et al., 2002; Hayashi et al., 2004; Sakai et al., 2007**). However, the entire biochemical pathway leading to 2PE has not yet been elucidated.

Tracer experiments in native plants with stable isotope-labeled precursors have long been used to discover unknown biochemical pathways (**Watanabe et al., 2002; Hayashi**

et al., 2004). However, several parameters such as feeding way, environmental factors, and difference between individual plants may influence the elucidation of biochemical pathway of target compounds and quantitative analysis. In particular, comparatively high feeding-concentrations of labeled precursors like amino acids and organic acids are used to enhance the visualization of target compounds, which may induce the damage of plant cell and hence influence the growth of plant (**Sayama, 2008**). Additionally, the dilution of fed isotope labeled compounds with the endogenous metabolite is often observed, resulting in the limitation to detect the intermediates of metabolic pathways (**Sayama, 2008**). Based on the above considerations, we developed a simple, accurate, and controllable approach to elucidate the biosynthesis of 2PE in rose using a protoplast-based system. In addition, we chemically synthesized a new stable isotope, [2,3,4,5,6-$^{13}C_5$] shikimic acid, to investigate the formation of 2PE from shikimic acid.

Results and discussion

Given low natural abundance and easy discrimination from natural metabolites, stable isotope-labeled precursors have long been used to tracer experiments for discovering unknown biochemical pathways (**Watanabe et al., 2002; Hayashi et al., 2004**). Therefore, we used this approach for the investigation of biosynthesis of 2PE in the protoplasts. Protoplasts isolated from rose petal exhibit the unique property of cell totipotency (**Davey et al., 2005**) and hence are able to continue producing and emitting volatile compounds after excision, which allowed us to use it as a model system for studying floral scent biosynthesis. Furthermore, protoplasts ("naked" cells) were

generated to ensure that there was no physical barrier from cell wall (**Davey et al., 2005**). This could provide an easy approach for intracellular metabolism of supplied precursors and also give a more uniform distribution of labeled precursor.

The first committed step in the biosynthesis of some volatile phenylpropanoid and benzenoid compounds is the deamination of L-Phe (**Dudareva and Pichersky, 2006**). Previously, we clarified that 2PE was synthesized from L-Phe by enzymatic systems consisting of aromatic amino acid decarboxylase and phenylacetaldehyde reductase (**Sakai et al., 2007**). Thus, in our labeling experiments we used deuterium labeled L-Phe ([$^2H_8$]-L-Phe) supplied to the protoplasts isolated from rose petals to investigate its metabolism. The end volatile products ([^2H_n]-2PE, n=6-8) were detected by GC-MS after supplying [$^2H_8$]-L-Phe. [^2H_n]-2PE (n=6-8) were determined from the MS chromatograms at *m/z* 130 / 98 ([M^+] / [M-C^2H_2O]$^+$) for [$^2H_8$]-2PE, *m/z* 129 / 97 ([M^+] / [M-C^2H_2O]$^+$) for [$^2H_7$]-2PE, and *m/z* 128 ([M^+]) for [$^2H_6$]-2PE. Since PAld is a key intermediate in the formation of some phenylpropanoid-related compounds from L-Phe (**Watanabe et al., 2002**), its presence in rose floral scent allowed us to follow its labeling pattern in the volatile fraction after supplying [$^2H_8$]-L-Phe. As a consequence, [^2H_n]-PAld (n=6-8) were determined from the MS chromatograms at *m/z* 128 / 100 / 99 / 98 ([M^+] / [M-C^2HO+^2H]$^+$ / [M-C^2HO+H]$^+$ / [M-C^2HO]$^+$) for [$^2H_8$]-PAld, *m/z* 127 / 98 / 97 ([M^+] / [M-CHO+H]$^+$ / [M-CHO]$^+$) for [$^2H_7$]- PAld, and *m/z* 126 / 97 / 96 ([M^+] / [M-CHO+H]$^+$ / [M-CHO]$^+$) for [$^2H_6$]- PAld. These observations are consistent with the formation of [^2H_n]-2PE (n=6-8) from [$^2H_8$]-L-Phe in our previous investigations on intact rose flowers (**Watanabe et al., 2002; Hayashi et al., 2004; Sakai et al., 2007**). Moreover, the quantitative analysis showed that treatment with [$^2H_8$]-L-Phe significantly increased the total amount of 2PE (p

< 0.05) in the protoplasts relative to the untreated group (control), which was due to the formation of [$^{2}H_{n}$]-2PE (n=6-8) from [$^{2}H_{8}$]-L-Phe (**Fig. 2A**). These results suggest that protoplasts could provide simple systems that may be developed as a viable alternative to the traditional use of native flower or excised petal tissue in studies on biosynthesis of 2PE.

The biochemistry and enzymology of floral scent have mainly concentrated on the isolation and characterization of enzymes and genes involved in the final steps of the biosynthesis of scent volatile compounds (**Boatright et al., 2004**). However, little is known about the entire biochemical pathway leading to simple volatile phenylpropanoids / benzenoids. To investigate the formation of 2PE from shikimate pathway, we supplied possible progenitor compounds, chorismic acid and shikimic acid (together with PEP) to the protoplasts isolated from rose petals. As a consequence, both treatments significantly increased the amount of 2PE in the protoplasts ($p < 0.01$, **Fig. 2A**) compared with the untreated group, indicating the formation of 2PE from chorismic acid and shikimic acid in the protoplasts. Then, we chemically synthesized ring-^{13}C labeled shikimic acid ([2,3,4,5,6-$^{13}C_5$] shikimic acid) (**Fig. 1**) supplied to the protoplasts to study 2PE derived from shikimate pathway, which guarantees that signals observed in the protoplast system was due to the incorporation of [2,3,4,5,6-$^{13}C_5$] shikimic acid. In the protoplasts fed with [2,3,4,5,6-$^{13}C_5$] shikimic acid, [2,3,4,5,6-$^{13}C_5$] 2PE was determined by GC-MS analysis (**Fig. 2B**), and the signals that due to ^{13}C in the aromatic rings of compound such as L-Phe were detected in NMR spectra, indicating a conversion of fed [2,3,4,5,6-$^{13}C_5$] shikimic acid to nonvolatile intermediate compounds with aromatic ring during the incubation period (24 h) (**Fig. 2C**). On the basis of the above observations from the

protoplast-based system, we propose the hypothetical biosynthetic pathway of 2PE from shikimic acid *via* chorismic acid, L-Phe, and PAld as depicted in **Fig. 3**. This information is helpful for the future discovery of enzymes and genes involved in the phenylpropanoids / benzenoid network, which still remain unknown in the rose flowers.

Phenylpropanoids metabolism comprises a complex series of branching biochemical pathways leading from L-Phe to nonvolatile aromatic compounds (e.g. flavonoids and anthocyanins) and volatile phenylpropanoids / benzenoids as common constituents of floral scent (**Boatright et al., 2004; Dudareva and Pichersky, 2006**). An advantage of the protoplast-based system generated here is to simplify the complex branching biosynthetic pathways of floral scent to distinct individual event, facilitating our understanding of the formation of floral scent. Although biosynthesis of floral volatiles has been investigated to a certain extent in floral tissues, very little information on the subcellular localization of respective enzymes and the compartmentalization of pathways is available (**Dudareva and Pichersky, 2006**). With the availability of subcellular fractions isolated from protoplast (**Davey et al., 2005**), the applicability of isolated protoplasts as a system for investigating the biosynthesis of floral volatiles would advance our understanding of the formation of floral volatiles.

Experimental

Chemicals

Shikimic acid, chorismic acid, phosphoenolpyruvate (PEP), and PAld were purchased

from Sigma-Aldrich Inc., U.S.A.. [a,b,b,2,3,4,5,6-$^{2}H_8$]-L-phenylalanine ([$^{2}H_8$]-L-Phe) (deuterium atom ≥ 98%) were purchased from Isotec Inc., U.S.A.. Cellulase "Onozuka" RS (20,000 units/g, cellulase derived from *Trichoderma viride*) and Macerozyme R-10 (3,000 units/g, enzyme derived from *Rhizopus* sp.) were purchased from Yakult Pharmaceutical Ind. Co. Ltd., Tokyo, Japan. Trypan-blue stain solution (0.5%) was purchased from Nacalai Tesque Inc., Kyoto, Japan. D-[U-$^{13}C_6$] mannose (^{13}C atom ≥ 98%) was purchased from Omicron Biochemicals Inc. U.S.A..

Synthesis of [2,3,4,5,6-$^{13}C_5$] shikimic acid

[2,3,4,5,6-$^{13}C_5$] shikimic acid was synthesized from D-[U-$^{13}C_6$] mannose according to the literatures (**Brimacombe et al., 1968; Fleet et al., 1984; Cho et al., 1992**) with modifications (**Fig. 1**, the detailed synthesis steps are described in **Supplementary Material**). The ^{1}H NMR (270 MHz) and ^{13}C NMR (67.5 MHz) spectra were measured with a JEOL JNM-EX270 NMR spectrometer. TMSi was used as an internal standard for the ^{1}H NMR and ^{13}C NMR spectra. [2,3,4,5,6-$^{13}C_5$] shikimic acid, ^{1}H-NMR (270 MHz, CD_3OD): δ1.95, 2.44 (1H, $^{1}J_{C\text{-}6,\ H\text{-}6}$= 138 Hz, H-6), 2.44, 2.93 (1H, $^{1}J_{C\text{-}6,\ H\text{-}6}$= 135 Hz, H-6), 3.40, 3.93 (1H, $^{1}J_{C\text{-}4,\ H\text{-}4}$= 143 Hz, H-4), 3.71, 4.24 (1H, $^{1}J_{C\text{-}3,\ H\text{-}3}$= 143 Hz, H-3), 4.09, 4.62 (1H, $^{1}J_{C\text{-}5,\ H\text{-}5}$= 143 Hz, H-5), 6.48, 7.09 (1H, $^{1}J_{C\text{-}2,\ H\text{-}2}$=163 Hz, H-2); ^{13}C-NMR (67.5 MHz, CD_3OD): δ31.4-31.9 (1C, dd, *J*=2.2 and 35.2 Hz, C-6), 66.7-68.9 (2C, m, C-5 and C-4), 72.2-73.4 (1C, dd, *J*=40.2 and 39.6 Hz, C-3), 130.7 (1C, s, C-1), 138.4-139.1 (1C, dd, *J*=2.8 and 44.7 Hz, C-2), 173.3 (1C, s, C-7); Remarkably high intensities on the ^{13}C-NMR signals of C-2, C-3, C-4, C-5, and C-6. HRMS (ESI) calcd

for $C_2{}^{13}C_5H_{10}O_5$ $[M-H]^-$ 178.0618. Found: 178.0616.

Preparation of protoplasts from rose petals and estimation of cell viability

The petals (1.5 g) of *Rosa* 'Yves Piaget' (from Ichikawa Rosary in Shizuoka, Japan), a cut rose cultivar that blooms throughout season, were sliced into 25-mm^2 pieces and placed immediately in the enzyme-digestion solution [200 mg of Cellulase "Onozuka" RS, and 100 mg of Macerozyme R-10 in 10 ml of protoplast buffer, which consists of 1.093 g of mannitol, 11.1 mg of calcium chloride, 2 ml of 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (100 mM, pH 5.6), and 8 ml of Milli-Q water]. The petal and enzyme mix was then aspirated for 3 min and incubated for 3.5 h at 25 °C in a water bath. After digestion, the petal tissues and cell debris were removed by nylon filter. Subsequently, the digestion mix was centrifuged at 100 *g* for 3 min to gently pellet protoplasts. After centrifugation, the supernatant was removed and the protoplasts were resuspended in protoplast buffer (10 ml). This operation was repeated by centrifugation, removal of supernatant, and adding 10 ml of protoplast buffer to pellet protoplasts. Following this, we removed as much supernatant as possible without removing suspended protoplasts, and 2 ml of protoplast buffer was added to give the protoplast solution.

The above protoplast solution (160 µl) was mixed with 0.5% Trypan-blue stain solution (40 µl). The resulting mix was loaded on a hematimeter (Thoma cell, Japan Clinical Instrument Ind. Co. Ltd., Tokyo, Japan). Total cell viabilities were estimated by a CH-A(B)-LB Biological Microscope (Olympus Co. Japan). **Fig. S1** (**Supplementary**

Material) shows the protoplast from rose petals (*Rosa* 'Yves piaget'). Cell viabilities of the protoplast treated with the precursor compounds used in the present study for 24 h were above 87%.

Labeling experiments

The above protoplast solution was incubated for 2 h at 30 °C in a water bath to emit the endogenous 2PE in the protoplasts. Subsequently, the protoplast solution was centrifuged at 100 *g* for 3 min. After centrifugation, the supernatant was removed and the protoplasts were resuspended in protoplast buffer (3 ml). The resulting protoplast solution was separated into 200 µl (4.0×10^5 cells) per treatment and then treated with the following solution, **treatment 1**) protoplast buffer (50 µl) as a control; **treatment 2**) 50 mM [$^2H_8$]-L-Phe in 50 µl of protoplast buffer; **treatment 3**) 50 mM chorismic acid in 50 µl of protoplast buffer; **treatment 4**) 100 mM shikimic acid in 25 µl of MES buffer (100 mM, pH 6.8) and 100 mM PEP in 25 µl of MES buffer (100 mM, pH 6.8); **treatment 5**) 100 mM [2,3,4,5,6-$^{13}C_5$] shikimic acid in 25 µl of MES buffer (100 mM, pH 6.8) and 100 mM PEP in 25 µl of MES buffer (100 mM, pH 6.8). All mixed solutions were incubated for 24 h at 30 °C in water bath, and 5 µl of ethyl decanoate in methanol (0.31 mM) was added as the internal standard. The resulting mix was extracted with 700 µl of hexane: ethyl acetate (1:1, v/v) twice. The organic fraction was dried over Na_2SO_4, and subjected to gas chromatography-mass spectrometry (GC-MS) analyses.

To detect PAld, the intermediate compound of biosynthesis of 2PE, a solution of 50 mM [$^2H_8$]-L-Phe in 50 µl MES buffer was supplied to the protoplast solution (200 µl) as

described above. The reaction solution was incubated for 3 h at 30 °C in water bath. Preparation of GC-MS analysis as described above was performed.

GC-MS conditions

Analyses of 2PE, [$^{2}H_{n}$]-2PE (n=6-8), (in **treatment 1-4** as shown in above), PAld, and [$^{2}H_{n}$]-PAld were performed using a GC-MS QP5050 (Shimadzu), which was controlled by a Class-5000 work station. For the analysis of 2PE and [$^{2}H_{n}$]-2PE (n=6-8), the GC was equipped with a capillary TC-WAX column (GL Sciences Inc., Japan), 30 m × 0.25 mm I.D., and 0.25 μm film thickness. For the analysis of PAld and [$^{2}H_{n}$]-PAld, the GC was equipped with a capillary TC-5 column (GL Sciences Inc., Japan), 30 m × 0.25 mm I.D., and 0.25 μm film thickness. The injector temperature was 230 °C. The GC oven was maintained at 60 °C for 3 min. The temperature of the oven was programmed at 15 °C/min to 110 °C, at 30 °C/min to 180 °C, at 40 °C/min to 290 °C, and kept at this temperature for 3 min. Helium was used as a carrier gas with a flow of 1.6 ml/min. The mass range of *m/z* 60-200 was operated in full scan mode. The ionizing voltage was70 eV, and the scanning speed was 0.5 scan/sec.

The GC-MS analysis of [2,3,4,5,6-$^{13}C_{5}$] 2PE (in **treatment 5** as mentioned above) 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 programmed from 60 °C (3 min hold), at 40 °C/min to 180 °C, at 10 °C/min to 240 °C, and kept at this temperature for 3 min. Other conditions were similar as described above.

NMR tracing of nonvolatile intermediates in the [2,3,4,5,6-$^{13}C_5$] shikimic acid labeled experiment

The protoplast solutions (200 μl) were treated with [2,3,4,5,6-$^{13}C_5$] shikimic acid (100 mM, 25 μl) and PEP (100 mM, 25 μl) for 0 h and 24 h respectively. The aqueous layer (100 μl) after extracting volatile components and D_2O (100 μl) were transferred into an NMR tube (3 mmϕ), and directly subjected to the ^{13}C- NMR analyses (JEOL JNM-LA 500 FT-NMR; proton decoupling; 125 MHz; 32768 data points; spectral width, 33898 Hz; acquisition time, 0.9667 sec; pulse delay, 2.033 sec; scans, 17177 times).

Statistical analysis

All experiments (except NMR studies) were performed in triplicate. One-way ANOVA was used to estimate overall significance followed by post hoc Turkey's tests corrected for multiple comparisons. Data are presented as mean ± S. D. A probability level of 5% ($p < 0.05$) was considered significant.

Acknowledgments

This work was supported in part by grant-aid to N. W. for scientific research (B) (2) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

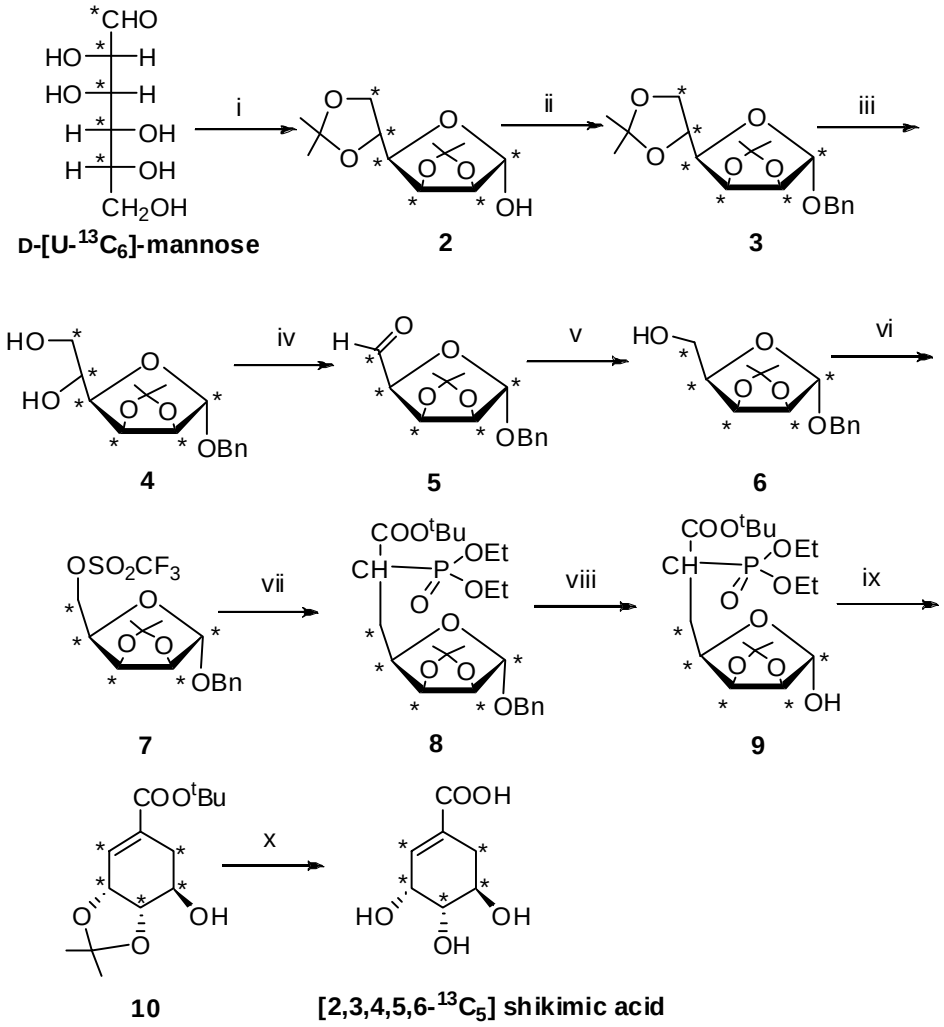
Legends to figures:

Figure 1 Synthesis of [2,3,4,5,6-$^{13}C_5$] shikimic acid

Figure 2 Production of 2PE and [$^{2}H_n$]-2PE derived from [$^{2}H_8$]-L-Phe, chorismic acid, and shikimic acid (together with PEP) supplied to the protoplasts (**A**), GC-MS profiles of [2,3,4,5,6-$^{13}C_5$] 2PE converted from [2,3,4,5,6-$^{13}C_5$] shikimic acid (**B**), and ^{13}C NMR spectra of the protoplasts fed with [2,3,4,5,6-$^{13}C_5$] shikimic acid for 0 h and 24 h respectively (**C**)

(A) Data are presented as mean ± S.D (n=3). N.S., non-significant, * $p < 0.05$, ** $p < 0.01$ when compared with the untreated protoplast group (CK). (B) Control, the untreated protoplasts; Treatment with [2,3,4,5,6-$^{13}C_5$] shikimic acid, the protoplasts treated with [2,3,4,5,6-$^{13}C_5$] shikimic acid for 24 h. (C) The protoplasts (200 μl) were treated with [2,3,4,5,6-$^{13}C_5$] shikimic acid (100 mM, 25 μl) and PEP (100 mM, 25 μl) for 0 h and 24 h respectively.

Figure 3 Hypothetical biosynthetic pathway for the formation of 2PE from shikimic acid.



*, ^{13}C label

i) Acetone, $FeCl_3$; ii) BnCl, NaH; iii) HCl, Methanol; iv) $NaIO_4$; v) $NaBH_4$; vi) $(CF_3SO_2)_2O$, Pyridine; vii) $(EtO)_2POCH_2COO^tBu$, NaH; viii) $HCOONH_4$, palladium/carbon; ix) NaH; x) 60% CF_3COOH

Fig. 1 Ziyin YANG

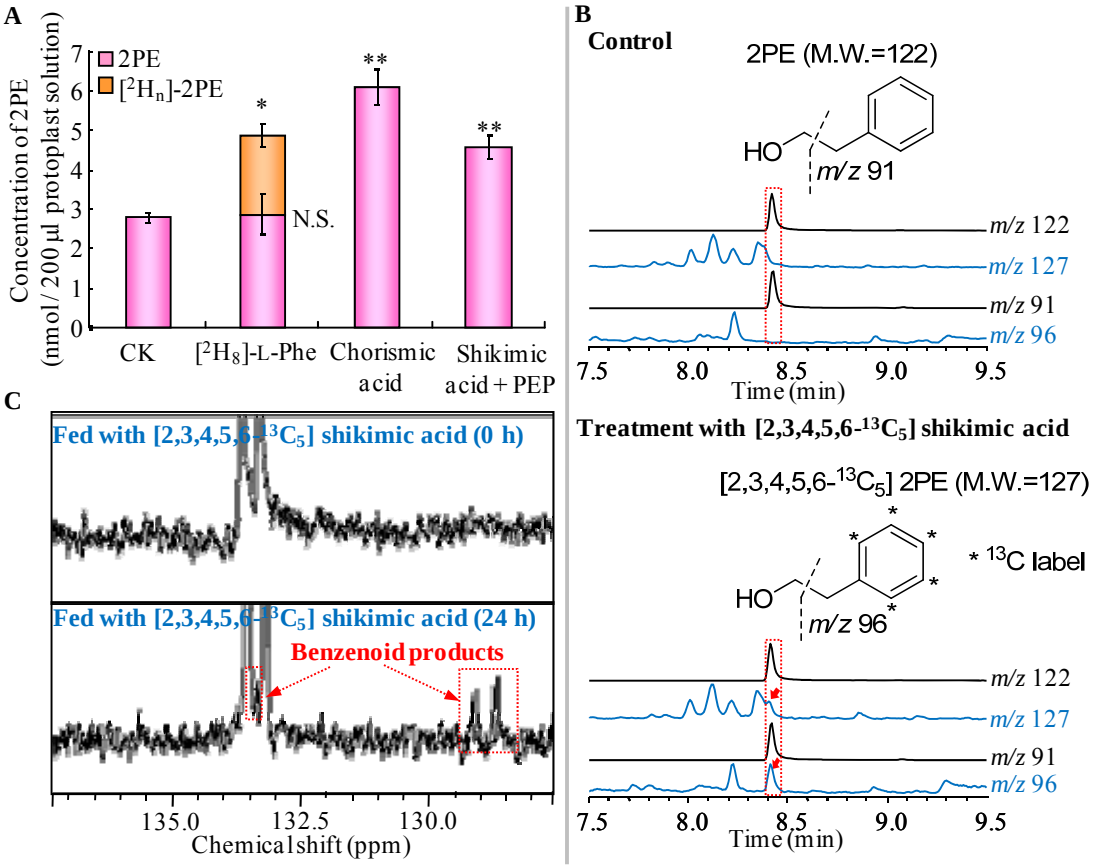


Fig. 2 Ziyin YANG

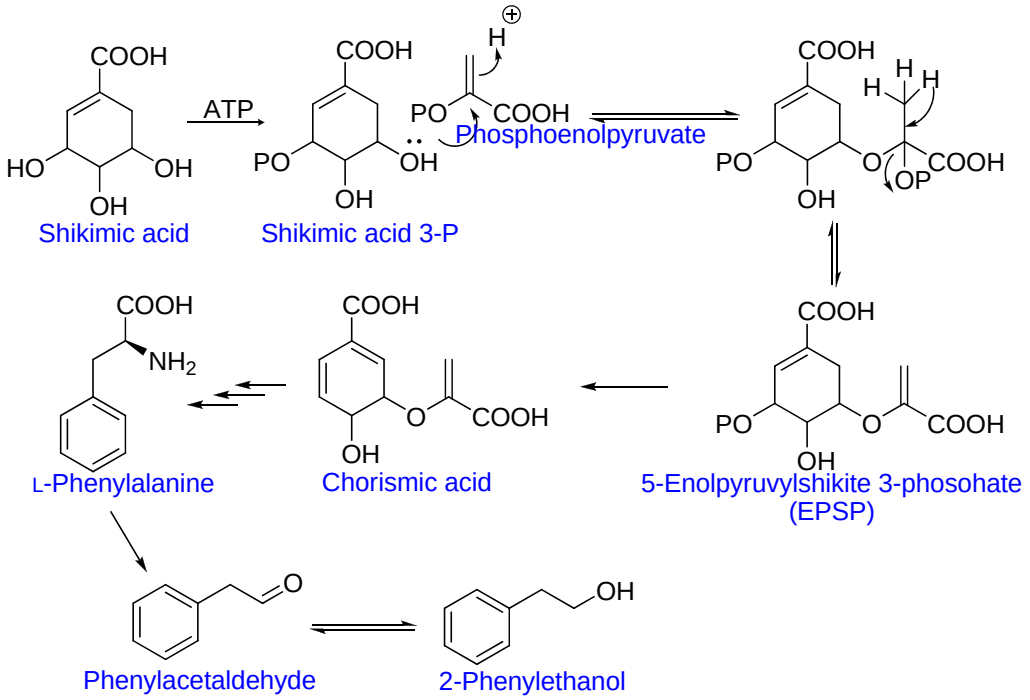


Fig. 3 Ziyin YANG

References

Baldermann, S., Yang, Z.Y., Sakai, M., Fleischmann, P., Watanabe, N., 2008. Volatile constituents in the scent of roses. Special Issue on Roses, in Global Science Books, Isleworth, UK, accepted.

Boatright, J., Negre, F., Chen, X.L., Kish, C.M., Wood, B., Peel, G., Orlova, I., Gang, D., Rhodes, D., Dudareva, N., 2004. Understanding in vivo benzenoid metabolism in petunia petal tissue. Plant Physiol. 135, 1-19.

Brimacombe, J.S., Hunedy, F., Tucker, L.C.N., 1968. Nucleophilic displacement reactions in carbohydrates. Part V. Reaction of 2, 3-*O*-isopropylidene-5-*O*-methylsulphonyl-D-lyxofuranose with sodium methoxide: aldehyde-group participation. J. Chem. Soc. C: Organic, 1381-1384.

Cho, H., Heide, L., Floss, H.G., 1992. Synthesis of D-(-)-[1,7-$^{13}C_2$]shikimic acid. J. Labelled Comp. Radiopharm. 31, 589-594.

Davey, M.R., Anthony, P., Power, J.B., Lowe, K.C., 2005. Plant protoplasts: status and biotechnological perspectives. Biotechnol. Adv. 23, 131-171.

Dewich, P.M., 2002. Medicinal natural products: A biosynthetic approach (second edition). John Wiley & Sons Ltd, England, pp. 121-166.

Dudareva, N., Pichersky, E., 2006. Floral scent metabolic pathways: their regulation and evolution. In: Dudareva, N., Pichersky, E. (Eds.), Biology of Floral Scent. Taylor & Francis Group, Boca Raton, London, New York, pp. 55-78.

Fleet, G.W.J., Shing, T.K.M., Warr, S.M., 1984. Enantiospecific synthesis of shikimic acid from D-mannose: formation of a chiral cyclohexene by intramolecular olefination of a

carbohydrate-derived intermediate. J. Chem. Soc. Perkin Trans. 1, 905-908.

Hayashi, S., Yagi, K., Ishikawa, T., Kawasaki, M., Asai, T., Picone, J., Turnbull, C., Hiratake, J., Sakata, K., Takada, M., Ogawa, K., Watanabe, N., 2004. Emission of 2-phenylethanol from its β-D-glucopyranoside and the biogenesis of these compounds from [$^{2}H_{8}$] L-phenylalanine in rose flowers. Tetrahedron 60, 7005-7013.

Pichersky, E., Noel, J.P., Dudareva, N., 2006. Biosynthesis of plant volatiles: Nature's diversity and ingenuity. Science 331, 808-811.

Sakai, M., Hirata, H., Sayama, H., Sekiguchi, K., Itano, H., Asai, T., Dohra, H., Hara, M., Watanabe, N., 2007. Production of 2-phenylethanol in roses as dominant floral scent compound from L-phenylalanine by two key enzymes: a PLP-dependent decarboxylase and a phenylacetaldehyde reductase. Biosci. Biotechnol. Biochem. 71, 2408-2419.

Sayama, H., 2008. Chemical synthesis of ^{13}C-shikimic acid and its metabolism in rose petals (*in Japanese*). Master Dissertation, Shizuoka University, Japan.

Watanabe, S., Hayashi, K., Yagi, K., Asai, T., MacTavish, H., Picone, J., Turnbull, C., Watanabe, N., 2002. Biogenesis of 2-phenylethanol in rose flowers: incorporation of [$^{2}H_{8}$] L-phenylalanine into 2-phenylethanol and its -D-glucopyranoside during the flower opening of *Rosa* 'Hoh-Jun' and *Rosa* damascena 'Mill'. Biosci. Biotechnol. Biochem. 66, 943-947.

Supplementary Material

Synthesis of [2,3,4,5,6-$^{13}C_5$] shikimic acid

*Synthesis of 2,3,5,6-di-O-isopropyrydene-α-D-[1,2,3,4,5,6-$^{13}C_6$] mannofuranose (**2**)*

D-[U-$^{13}C_6$] mannose (1.0 g, 5.7 mmol) was dissolved in dry acetone (20 ml) and $FeCl_3$ (50 mg, 0.3 mmol) was added to the solution. The reaction solution was heated to 100 °C under reflux for 30 min. The resulting organic layer was washed by 10% aqueous K_2CO_3 at room temperature, and then heated to remove acetone. Subsequently, the solution was extracted with CH_2Cl_2 (3 × 20 ml). The CH_2Cl_2 fraction was dried over Na_2SO_4, and concentrated. The residual oil was purified using silica gel column chromatography with 0%→50%→70% ethyl acetate in hexane to give compound **2** as white crystal (1.34 g, 5.0 mmol, 87.7%). ^{1}H-NMR (270 MHz, $CDCl_3$): δ 1.23-1.46 (12H, m, CH_3), 2.67 (1H, d, *J*=9.1 Hz, HO-1) , 4.08-4.16 (2H, m, H_2-6), 4.34-4.36 (1H, dd, *J*=3.6 and 7.2 Hz, H-3), 4.44-4.49 (1H, m, H-2), 4.89-5.02 (1H, m, H-5), 5.04-5.07 (1H, m, H-4), 5.67-5.71 (1H, m, H-1); ^{13}C-NMR (67.5 MHz, $CDCl_3$): δ 24.0-26.0 (4C, s, CH_3), 66.2-66.8 (1C, dd, *J*=1.7 and 34.3 Hz, C-4), 72.6-73.8 (1C, m, C-5), 78.9-81.0 (2C, m, C-3 and C-6), 84.8-86.0 (1C, m, C-2), 100.8-101.6 (1C, m, C-1).

*Synthesis of benzyl 2,3,5,6-di-O-isopropyrydene-α-D-[1,2,3,4,5,6-$^{13}C_6$] mannofuranosisede (**3**)*

Benzyl chloride (1.5 ml) was added to a solution of 60% sodium hydride (200 mg, 0.2 mmol) in dry *N,N*-dimethylformamide (DMF, 2.5 ml). Subsequently, a solution of

compound **2** (1.04 g, 3.9 mmol) in dry DMF (2.5 ml) was added to the above solution. The mixture was stirred for 12 h at room temperature, and then methanol (6 ml) was added to the mixture to quench the reaction. The solution was heated to 100 °C under reflux for 30 min. After cooling to room temperature, water (25 ml) was added to the solution. Subsequently, the solution was extracted with ethyl acetate (3 × 20 ml). The organic fraction was dried over Na_2SO_4, and concentrated. The residual oil was purified using silica gel column chromatography with 0%→10%→20%→50% ethyl acetate in hexane to give compound **3** as white crystal (969 mg, 2.7 mmol, 69.7%). ^{1}H-NMR (270 MHz, $CDCl_3$): δ1.23-1.46 (12H, m, CH_3), 3.95-4.00 (2H, m, H_2-6), 4.08-4.13 (1H, dd, *J*=6.6, and 8.9 Hz, H-3), 4.37-4.44 (1H, dddd, *J*=8.9, 6.6, 5.9, and 1.9, H-4), 4.46-4.51 (1H, d, *J*=6.6 Hz, H-2), 4.62-4.66 (2H, t, *J*=5.77 Hz, Ph-C$\underline{H}_2$), 4.78-4.81 (1H, dd, *J*=3.6, and 5.9, H-5), 5.07 (1H, s, H-1), 7.19-7.35(5H, m, Ph); ^{13}C-NMR (67.5 MHz, $CDCl_3$): δ24.0–26.0 (4C, s, CH_3), 66.7-67.4 (1C, m, C-2), 68.8-69.4 (1C, m, C-5), 72.9-80.5 (3C, m, C-3, C-6, and Ph–C$\underline{H}_2$), 84.6-85.5 (1C, m, C-2), 105.5-106.8 (1C, m, C-1), 127-137 (6C, m, Ph).

*Synthesis of benzyl 2,3-O-isopropyrydene-α-D-[1,2,3,4,5,6-$^{13}C_6$] mannofuranosisede (**4**)*

Compound **3** (872 mg, 2.44 mmol) was dissolved in methanol (8 ml), and 36.5% HCl (120 μl) was dripped in the solution during 1 min. The resulting mixture was diluted with water to turbidity, and stirred for 12 h at room temperature. The solution was neutralized with aqueous $NaHCO_3$ to pH 7.0 and extracted with ethyl acetate (3 × 20 ml). The organic fraction was dried over Na_2SO_4, and concentrated. The residual crude crystal was purified using silica gel column chromatography with 0%→10%→20%→30% →40%

→50%→60%→100% ethyl acetate in hexane and 100% methanol to give compound **4** as white crystal (689 mg, 2.2 mmol, 89.3%). ^{1}H-NMR (270 MHz, $CDCl_3$): δ 1.23-1.32 (6H, m, CH_3), 3.66-3.73 (1H, m, H-5), 4.21-4.38 (2H, m, H-3 and H-4), 4.08-4.16 (2H, dd, *J*=7.1 and 14.3 Hz, H_2-6), 4.48-4.65 (2H, m, Ph-C$\underline{H}_2$), 4.77-4.96 (1H, m, H-2), 5.14-5.45 (1H, m, H-1), 7.26-7.38(5H, m, Ph); ^{13}C-NMR (67.5 MHz, $CDCl_3$): δ 22.0-26.0 (2C, s, CH_3), 64.1-64.7 (1C, dt, *J*=1.6, and 20.9 Hz, C-6), 69.8-71.0 (1C, m, C-4), 78.6-80.7 (2C, m, C-3 and C-2), 84.2-85.4 (1C, m, C-5), 105.1-105.8 (1C, dd, *J*=3.91, and 49.1 Hz, C-1).

*Synthesis of benzyl 2,3-O-isopropyrydene-α-D-[1,2,3,4,5-$^{13}C_5$] lyxofuranoside (**6**)*

Compound **4** (683 mg, 2.16 mmol) was dissolved in methanol (14 ml), and a solution of $NaIO_4$ (520 mg) in minimum amount of water was dripped to the solution. The resulting mixture was stirred for 2.5 h at room temperature, and then evaporated to remove the solvent. The residual oil was dissolved in acetone (10 ml), and filtrated. The resulting filtrate was concentrated to give compound **5** as colorless oil. Subsequently, compound **5** was dissolved in ethanol (14 ml), and $NaBH_4$ (100 mg) was added to the solution. The resulting mixture was stirred for 2 h at room temperature, and then ethyl acetate (2 ml) was added to quench the reaction. The solution was evaporated to remove solvent, and the residual solid was dispersed with water (15 ml). The aqueous solution was extracted with diethyl ester (3 × 20 ml). The organic fraction was dried over Na_2SO_4, and concentrated. The residual crude crystal was purified using silica gel column chromatography with 0%→50%→70%→100% ethyl acetate in hexane to obtain compound **6** as white crystal (540 mg, 1.9 mmol, 87.7%). ^{1}H-NMR (270 MHz , $CDCl_3$) :

δ1.30 (3H, s, CH_3), 1.46 (3H, s, CH_3), 3.67-3.84 (2H, m, H_2-5), 4.18-4.39 (2H, m, H-3 and H-4), 4.48-4.65 (2H, m, Ph-C$\underline{H_2}$), 4.93-4.98 (1H, m, H-2), 5.11-5.48 (1H, m, H-1), 7.25-7.38(5H, m, Ph); ^{13}C-NMR (67.5 MHz, $CDCl_3$): δ24.4-25.8(2C, s, CH_3), 60.7-61.3 (1C, m, C-5), 78.7-80.9 (2C, m, C-3 and C-2), 84.6-85.8 (1C, m, C-4), 104.8-105.6 (1C, dd, *J*=3.91, and 49.7 Hz, C-1).

*Synthesis of benzyl 2,3-O-isopropyrydene-5-O-trifluoromethylsulphonyl-α-D-[1,2,3,4,5-$^{13}C_5$] lyxofuranoside (**7**)*

Compound **6** (309 mg, 1.08 mmol) was dissolved in dry dichloromethane (20 ml), and dry pyridine (169 mg, 2.15 mmol) was added to the solution. Trifluoromethanesulphonic acid anhydride (400 mg, 1.4 mmol) was added dropwise to the above mixture during 5 min at -30 °C under Ar. The solution was stirred for 15 min at -30 °C, and then methanol (1 ml) was added to quench the reaction. The solution was sufficiently washed with ice water (20 ml), and aqueous KH_2PO_4 (1M, 20 ml) successively. The organic fraction was dried over Na_2SO_4, and concentrated. The residuals with toluene were evaporated to remove pyridine. The resulting colorless oil was purified using silica gel column chromatography with 0%→50%→70%→100% ethyl acetate in hexane to obtain compound **7** as colorless oil (427 mg, 1.02 mmol, 94.3%). ^{1}H-NMR (270 MHz, $CDCl_3$) : δ1.29 (3H, s, CH_3), 1.44 (3H, s, CH_3), 4.18-4.52 (2H, m, H_2-5), 4.32-4.70 (2H, m, H-3 and H-4), 4.75-4.81 (3H, m, H-2 and Ph-C$\underline{H_2}$), 5.11-5.29 (1H, m, H-1), 7.25-7.39 (5H, m, Ph).

Synthesis of tert-butyl (3R,4S,5R)-3,4,5-trihydroxy-3,4-O-isopropylidene-

*[2,3,4,5,6-$^{13}C_5$] cyclohex-1-enecarboxylate (**10**)*

60% NaH (66 mg, 2.75 mmol) was dissolved in dry DMF (5 ml), and *tert*-butyl diethoxyphosphorylacetate (375 mg, 1.49 mmol) was added dropwise to the solution during 20 min at 0 °C. The mixture was stirred to become clear at room temperature. A solution of compound **7** (427 mg, 1.02 mmol) in DMF (2 ml), and 18-crown-6 (1 drop) were added to the mixture. The solution was stirred for 20 h at room temperature, and then cooled to 0 °C. Subsequently, aqueous KH_2PO_4 (1 M, 10 ml) was added to quench the reaction. The solution was extracted with $CHCl_3$ (3 × 20 ml). The organic fraction was dried over Na_2SO_4, and concentrated. The residual yellow oil was purified using silica gel column chromatography to give compound **8** (202 mg, 0.39 mmol, 38.2%) as yellow oil. Subsequently, compound **8** (202 mg, 0.39 mmol) was dissolved in methanol (18 ml), and then 10% palladium/carbon (152 mg) was added to the solution. The mixture was stirred for 1 h at 75 °C, and cooled to room temperature, and then filtered with Celite and the filtrate was concentrated to give compound **9** (158 mg, 0.37 mmol) as yellow oil. Subsequently, a solution of compound **9** (158 mg, 0.37 mmol) in dry tetrahydrofuran (THF) (2 ml) was added dropwise to THF (5 ml) solution of 60% NaH (20 mg, 0.83 mmol) during 5 min, and then stirred for 45 min at room temperature. The mixture was cooled to 0 °C, and aqueous KH_2PO_4 (1 M, 10 ml) was added to quench the reaction. The solution was extracted with $CHCl_3$ (3 × 15 ml). The organic fraction was dried over Na_2SO_4, and concentrated. The residual colorless oil was purified using silica gel column chromatography with 0%→50%→70%→100% ethyl acetate in hexane to give compound **10** (28 mg, 0.10 mmol, 25.6%) as colorless oil. ^{1}H-NMR (270 MHz, $CDCl_3$): δ 1.41 (6H, s, CH_3), 1.47 (9H, s, ^{t}Bu), 2.11-2.22 (1H, dd, J=8.9, and 8.5 Hz, H-6),

2.75-2.83 (1H, dd, *J*=4.9, and 8.5 Hz, H-6), 3.79-3.87 (1H, ddd, *J*=8.9, 4.9, and 4.6 Hz, H-5), 4.02-4.08 (1H, dd, *J*=2.6, and 6.5 Hz, H-4), 6.84-6.86 (1H, broad, H-3), 7.26 (1H, t, *J*=2.63 Hz, H-2); ^{13}C-NMR (67.5 MHz, $CDCl_3$): δ 25.7 (2C, s, CH_3), 28.0 (3C, s, ^{t}Bu), 29.3-30.1 (1C, m, C-6), 68.4-69.3 (1C, m, C-5), 71.9-72.4 (1C, m, C-3), 78.0-78.6 (1C, m, C-4), 81.2 (1C, s, $(CH_3)_3\underline{C}$-), 109.6 (1C, s, $(CH_3)_2\underline{C}$-), 129.4-132.8 (1C, m, C-1 and C-2), 165.1(1C, s, C-7).

Synthesis of D*-(-)-[2,3,4,5,6-$^{13}C_5$] shikimic acid*

Compoud **10** (28 mg, 0.10 mmol) was dissolved in 60% aqueous trifluoroacetic acid (3 ml), and stirred for 36 h at room temperature, and then evaporated to remove solvent. The resulting colorless oil was purified using silica gel column chromatography with 0%→50%→70%→100% ethyl acetate in hexane to give D-(-)-[2,3,4,5,6-$^{13}C_5$] shikimic acid (14 mg, 0.08 mmol, 79.2%) as colorless crystalline compound.

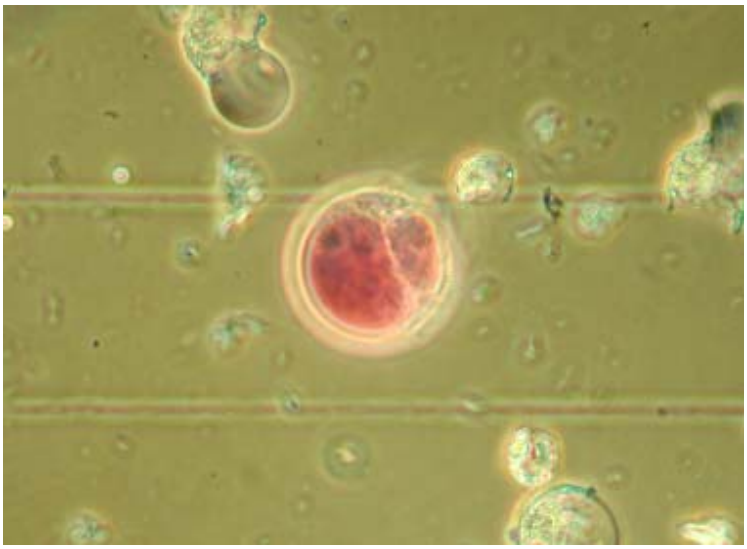


Fig. S1 Protoplast from rose petals (*Rosa* ‘Yves piaget’)