Functional characterization of rose phenylacetaldehyde reductase (PAR), an enzyme involved in the biosynthesis of the scent compound 2-phenylethanol

著者	Chen Xiao-Min, Kobayashi Hiromi, Sakai Miwa, Hirata Hiroshi, Asai Tatsuo, Ohnishi Toshiyuki, Susanne Baldermann, Watanabe Naoharu
journal or publication title	Journal of Plant Physiology
volume	168
number	2
page range	88-95
year	2011-01-15
出版者	Elsevier
権利	Copyright (C) 2010 Elsevier GmbH All rights reserved.
URL	http://hdl.handle.net/10297/5655

doi: 10.1016/j.jplph.2010.06.011

**Functional characterization of rose
phenylacetaldehyde reductase (PAR), an enzyme
involved in the biosynthesis of the scent compound 2-
phenylethanol**

**Xiao-Min Chen,¹ Hiromi Kobayashi,² Miwa Sakai,³ Hiroshi Hirata,¹
Tatsuo Asai,² Toshiyuki Ohnishi,⁴ Susanne Baldermann,¹ and
Naoharu Watanabe^{2*}**

¹Graduate School of Science and Technology, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

²Faculty of Agriculture, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

³The United Graduate School of Agricultural Science, Gifu University,
836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

⁴Division of Global Research Leaders, Shizuoka University,
836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.

* Corresponding author. Tel/Fax: +81-54-238-4870

E-mail address: acnwata@agr.shizuoka.ac.jp (N. Watanabe)

Summary

2-Phenylethanol (2PE) is a prominent scent compound released from flowers of Damask roses (*Rosa* ×*damascena*) and some hybrid roses (*Rosa* ‘Hoh-Jun’ and *Rosa* ‘Yves Piaget’). 2PE is biosynthesized from L-phenylalanine (L-Phe) via the intermediate phenylacetaldehyde (PAld) by two key enzymes, aromatic amino acid decarboxylase (AADC) and phenylacetaldehyde reductase (PAR).

Here we describe substrate specificity and cofactor preference in addition to molecular characterization of rose-PAR and recombinant PAR from *R. ×damascena*. The deduced amino acid sequence of the full-length cDNA encoded a protein exhibiting 77% and 75% identity with *Solanum lycopersicum* PAR1 and 2, respectively. The transcripts of *PAR* were higher in petals than calyxes and leaves and peaking at the unfurling stage 4. Recombinant PAR and rose-PAR catalyzed reduction of PAld to 2PE using NADPH as the preferred cofactor. Reductase activity of rose-PAR and recombinant PAR were higher for aromatic and aliphatic aldehydes than for ketocarbonyl groups. The both PARs showed that [4*S*-²H] NADPH was preferentially used over the [4*R*-²H] isomer to give [1-²H]-2PE from PAld, indicating that PAR can be classified as short-chain dehydrogenase reductase (SDR).

KEYWORDS

Enantio-selectivity; Phenylacetaldehyde reductase; 2-Phenylethanol; *Rosa* ×*damascena*; Recombinant enzyme; Substrate specificity

Abbreviations

AADC, Aromatic amino acid decarboxylase; PAld, Phenylacetaldehyde; PAR, Phenylacetaldehyde reductase; 2PE, 2-Phenylethanol; L-Phe, L-Phenylalanine.

1 **Introduction**

2 2-Phenylethanol (2PE) is a volatile compound with a pleasant fruity, floral
3 odor and is a major constituent of rose-like flowers scents. For example, 2PE
4 occupies 60% of the total volatiles in the essential oil of Damask roses (Rusanov et al.,
5 2005). Fruits, vegetables and foods such as cheese, bread, wine, and olive oil contain
6 2PE as a major flavor compound (Lee and Richard, 1984; Rodopulo et al., 1985;
7 Clark, 1990; Jollivet et al., 1992; Gassenmeier and Schieberle, 1995). Cosmetics
8 industry uses a large amount of 2PE as ingredients in perfume and other formulations
9 because of its popular rose-like smell (Clark, 1990; Fabre et al., 1998). Esters of 2PE,
10 especially phenylethyl acetate, are also valuable fragrance compounds (Bauer et al.,
11 2001). Increasing demand for natural flavors has led to a growing interest in
12 industrial-scale 2PE biosynthesis. Under US Food and Drug Administration products
13 derived from biotechnological processes can be labeled as “natural” based on US
14 Food and Drug Administration or regulations (Serra et al., 2005). 2PE also has
15 important biological functions in plants, such as antimicrobial properties (Berrah et al.,
16 1962) and reproduction *via* its attraction of pollinating insects (Pichersky and
17 Gershenzon, 2002). Therefore studies on regulation of 2PE biosynthesis and its
18 emission are very important subjects to be clarified. As a consequence, there has
19 been much interest in the biosynthesis pathway of 2PE in plants, as well as in bacteria
20 and yeast.

21 The rose 2PE biosynthetic pathway was at one time thought to convert L-
22 phenylalanine (L-Phe) *via* phenylpyruvate and phenyl acetic acid (Bugorskii and
23 Zaprometov, 1978). We demonstrated that L-Phe is a precursor of 2PE in rose
24 flowers using feeding experiments with labeled [²H] L-Phe (Watanabe et al., 2002;
25 Hayashi et al., 2003). Recently, a specific enzyme PAAS (phenylacetaldehyde
26 synthase) involved in the conversion of L-Phe to phenylacetaldehyde (PAld) was
27 isolated and characterized from *Petunia hybrida* cv. Mitchell (Kaminaga et al., 2006).
28 This PAAS belongs to group II pyridoxal 5'-phosphate-dependent amino acid
29 decarboxylases (AADCs). The AADC responsible for conversion of L-Phe to PAld
30 and also the first two rose-derived phenylacetaldehyde reductases (PAR) found to
31 catalyze the conversion of PAld to 2PE, which is the final reduction step of the
32 biosynthetic pathway, have yet been characterized in *Solanum lycopersicum* (AADC1,
33 AADC2, PAR1, PAR2) (Tieman et al., 2006, 2007). The *Solanum lycopersicum*

PAR1 is a member of short-chain dehydrogenase/reductase family, strongly prefers PAld as substrate and does not catalyze the reverse reaction however *Solanum lycopersicum* PAR2 has similar affinities for PAld, benzaldehyde and cinnamaldehyde. The contribution of AADC generating PAld from L-Phe, and PAR in the biosynthesis of 2PE via the intermediate PAld has been confirmed in *R. 'Hoh-Jun'* (Sakai et al., 2007). Recently, the function of PAAS has been confirmed by application of a *Saccharomyces cerevisia aro10Δ* mutant (Farhi et al., 2010).

To elucidate biochemical functions and molecular biological properties of rose-PAR involved in biosynthesis of 2PE, we characterized these properties. We purified a rose-PAR from *Rosa ×damascena* (Mabberley, 2008) and obtained partial peptide sequences based on TOF-MS analysis. We demonstrated that the full length cDNA encodes a functional PAR. Enzymatic analysis showed that the rose-PAR prefers PAld, but also converts several aldo- and keto-compounds. The recombinant PAR and rose-PAR showed similar substrate utilizing properties, however higher turnover rates were shown in the recombinant PAR with several substrates. Furthermore, the classification of PAR was discussed for rose-PAR and recombinant PAR based on the stereo-selectivity toward *S*- and *R*-[4-²H] NADPH.

Material and methods

Plant materials

Damask roses (*Rosa ×damascena* Mill.) were grown at the Field Research Center, Faculty of Agriculture, Shizuoka University, Japan. Flowers at stage 2 (Sepals haven't started to loosen, petals completely closed), stage 4 (outer whorl of petals is fully open, inner whorl starts to loosen) and stage 6 (petals are fully open, stamens are invisible; *ibid.*) were collected between April and May (2004-2009) (Hayashi et al., 2004). Flowers at stages 2, 4, and 6 and leaves were applied for transcripts expression experiments, and flowers at stage 4 were utilized for all the enzyme experiments.

Partial purification of rose-PAR

Floral extracts were prepared as described by Sakai et al. 2007. Briefly, flowers were homogenized in chilled buffer A (10 mM potassium phosphate buffer (pH 8.0), 5 mM DTT, 0.05% CHAPS, and 1% glycerol, 4 °C) and after centrifugation

(4000 g, 20 min, 4 °C) the resulting crude cell extracts were applied to ECONO pack Q cartridges (5 mL, Bio-Rad). Enzymatic active fractions were eluted with a linear gradient of 0-1 M KCl in buffer A at a flow rate of 1.5 mL min⁻¹. Fractions with enzyme activity were salted out with 150 mM KCl and equilibrated in buffer A. The diluted solutions were applied to two in-line blue HP columns (1 mL, GE Healthcare) equilibrated with buffer A. After washing the column with buffer A, enzymatic active fractions were eluted with a gradient of 0-150 mM KCl in buffer A at a flow rate of 1 mL min⁻¹. The gradient was 100-120 mM KCl within 5 min, 120-150 mM KCl within 3 min, and maintained for 5 min. PAR-containing fractions were used for the functional analysis. For sequencing the PAR fractions were combined and concentrated by centrifugal filtration (Nanosep 10 K, PALL Life Science) before application (200 µL) to a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with buffer A. The enzyme was eluted with 5 mL 150 mM KCl at a flow rate of 0.5 mL min⁻¹. The proteins were separated on the SDS-PAGE (12% acryl amide) and rose-PAR was detected at 35 kDa after Coomassie Brilliant Blue staining.

Molecular mass and partial amino acid sequence of PAR

The partial purified PAR enzyme was further purified by SDS-PAGE. Target bands detected at ca 35 kDa were excised and digested to peptides with trypsin for LC/MS/MS analysis (LC: Waters Nano Acquity, MS/MS: Waters-Micromass Q-ToF Premier). Five micro-liter of digest solution were injected and desalted on a trap column (0.18 × 20 mm, Nano Acquity, Waters) at a flow rate of 4 µL min⁻¹ with solvent A (0.1% formic acid) for 3 min. The peptides were separated on a C18 column (75 µm × 100 mm, Nano Acquity UPLC Beh, Waters). A linear gradient was developed 0-1 min: 3% solvent B (acetonitrile, 0.1% formic acid), 30 min: 40% B, 32-37 min: 95% B, 37 min: 95% B, 39 min: 3% B at a flow rate of 300 nL min⁻¹. The column temperature was 35 °C. The Q-TOF spectrometer was operated in the data dependent acquisition (DDA) mode using an ESI(+) MS survey scan on two different precursor ions. The peptide masses and sequences obtained were either matched automatically to proteins in the non-redundant database (NCBI) using the Mascot MS/MS ions search algorithm ([http:// www.matrixscience.com](http://www.matrixscience.com)).

Molecular cloning of PAR from *Rosa ×damascena* Mill.

Total RNA was isolated from the flower petals of *R. ×damascena* with a RNeasy Plant Mini Kit (QIAGEN). First strand cDNA was synthesized with AMV Reverse Transcriptase XL and Oligo dT- Adaptor Primer (TaKaRa RNA Kit 3.0). Full-length sequences of *PAR* in rose were obtained using degenerate primers designed from the amino acid sequences (No.1-3, Table 1). 3'-RACE PCR reactions were performed using 3'RACE-F1, 3'RACE-F2 and 3'RACE-F3 as forward primers (Supplementary Table 1). Amplified cDNAs were inserted into pCR 2.1 vector (Invitrogen) and transformed into DH5 α competent cells (TaKaRa). Isolated cDNA was sequenced using a Thermo Sequenase cycling sequencing kit (USB Corporation) on a LI-COR DNA sequencer (Model 4200L, Li-COR).

A 5'-RACE system kit was used for amplification of 5' ends (Invitrogen). The gene-specific primers (GSP) for 5'-RACE amplifications were designed based on the sequences obtained by 3'-RACE reactions (Supplementary Table 1). Reverse transcription from total RNA was performed using 5'-end-phosphorylated primer (GSP1) and SuperScript™ II (5' RACE System for Rapid Amplification of cDNA Ends, Invitrogen). The first PCR was performed using GSP2 primer and the abridged Anchor Primer (Invitrogen). Nested PCR was then performed using the GSP3 primer and Abridged Universal Amplification Primer (AUAP). Finally, end-to-end PCR was performed using PAR-F-1 as forward primer and PAR-R-1 as reverse primer (Supplementary Table 1). Nucleotide sequences were subsequently determined as described previously.

Expression and purification of recombinant PAR protein

*Bam*HI and *Sal*I sites were created on the 5' and 3'-ends of *PAR* by PCR using the primers PAR-F-E and PAR-R-E, respectively. The engineered cDNA fragments were inserted into the *Bam*HI-*Sal*I sites of pGEX-4T-1 (GE Healthcare), resulting in a recombinant gene product with an *N*-terminal glutathione *S*-transferase (GST) protein tag. Freshly transformed BL21 cells harboring pGEX-PAR or an empty pGEX vector were grown at 37 °C in 50 mL LB broth with 25 μ g mL⁻¹ ampicillin to an O.D.₆₀₀ = 0.6. 2.5 mL of the liquid culture were transferred to 250 mL LB broth containing the appropriate antibiotics and grown until O.D.₆₀₀ = 0.8 at 37 °C. 250 μ L of 1 mM IPTG solution were then added to induce production of the recombinant protein and the cultures grown another 8 h at 37 °C until an O.D.₆₀₀ = 1.8. The cells were harvested

by centrifugation (8000 g, 10 min, 4 °C) and after addition of 12.5 mL PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄ (pH 7.3)) the samples were frozen at -80 °C. All protein purification steps were carried out at 4 °C. The cells suspended in PBS were disrupted for 10 s 5 times by ultrasonication (UD201, TOMY). After the addition of 1% Triton X-100, the samples were centrifuged at 7700 g for 10 min to remove cell debris. Recombinant proteins were purified from the supernatant on GSTrap FF columns (5mL, GE Healthcare). GST tags were removed by on-column thrombin digestion (100 units, 2 h, room temperature) (GE Healthcare) and the enzyme was eluted with PBS. Thrombin was removed on a HiTrap Benzamidine FF column (GE Healthcare). The purity of the recombinant protein was analyzed by SDS-PAGE (12% acryl amide) as described previously (Fleischmann et al., 2003). A single protein was detected at 35 kDa after Coomassie Brilliant Blue staining (Supplementary Fig. 3). The recombinant PAR encoding the endogenous rose-PAR was subjected to functional analysis.

Determination of changes in transcripts of PAR in *Rosa ×damascena* Mill.

Total RNA was extracted using Fruit-mate (TaKaRa) and purified with Fastpure RNA kit (TaKaRa) followed by DNase treatment (Fermentas) to remove any contaminating DNA. First-strand cDNA was synthesized from 50 ng of total RNA by PrimeScript RT reagent Kit, Perfect Real Time (TaKaRa). Rose-PAR mRNA levels in petals, calyxes, and leaves were measured by real time quantitative RT-PCR. The real time RT-PCR reactions were performed utilizing the SYBR-Green I dye (SYBR Premix Ex Taq, Perfect Real Time, TaKaRa). The quantification was achieved from dose-response curves using β -tubline as an internal control in triplicate. Primers for real time RT-PCR (PAR-Q and TUB-Q) were described in Supplementary Table 1.

Aldehyde and ketone selectivity

Activities of rose-PAR and recombinant PAR were assayed at 30 °C by measuring the decrease in absorbance of NADPH at 340 nm ($\epsilon_{340} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$, Ultrospec 3000, Pharmacia Biotech) (Larroy et al., 2002). The reaction mixture (200 μ L) (100 mM potassium phosphate (pH 7.0), recombinant enzyme (6.8 μ g) /rose-PAR (8.0 μ g), 10 mM PAld, and 2.5 mM NADPH) was incubated at 30 °C for 10 min. The reaction was quenched by the addition of 300 μ L acetonitrile and centrifuged at

3000 g for 5 min. The relative activities of rose-PAR and recombinant PAR with selected substrates (Table 2) were determined by measuring the decrease in absorbance of NADPH at 340 nm using 10 mM of each substrate. Reaction conditions were the same as described for the PAR assay. One unit of enzyme activity was defined as the oxidation of 1 μmol NADPH min^{-1} at 30 °C. Specific activity was expressed as units /mg protein which was 10.1 mU mg^{-1} for rose-PAR and 0.7 mU mg^{-1} for recombinant PAR.

Synthesis of *S*-[4- ^2H] NADPH and *R*-[4- ^2H] NADPH

S-[4- ^2H] NADPH was synthesized from NADP^+ (0.019 mM) and [1- ^2H] glucose (0.08 mM) with 8 units of glucose dehydrogenase (*Bacillus* sp., Wako Pure Chemical) in 2 mL buffer (100 mM potassium phosphate and 0.1 mM EDTA, pH 8.0) at 37 °C for 1 h, and then maintained at 60 °C for 10 min (McCracken et al., 2004). Deuterated NADPH was isolated by HPLC with 1 mL min^{-1} flow rate at room temperature in gradient mode from 0%-100% B within 30 min using 25 mM phosphorous potassium buffer (pH 7.0) and 25 mM phosphorous potassium buffer (pH 7.0) and 0.5 M NH_4HCO_3 as the mobile phases (McCracken et al., 2004). *S*-[4- ^2H] NADPH was obtained by a column chromatography on negative ion exchange resin (HiTrap Q FF, GE Healthcare), and the concentration was determined based on the absorbance at 260 nm.

R-[4- ^2H] NADPH was synthesized using the stereo-specificity of alcohol dehydrogenases (McCracken et al., 2004). NADP^+ (0.022 mM) and [$^2\text{H}_8$] isopropanol (0.6 mM) were added to 7 mL of 25 mM Tris-HCl buffer (pH 9.0). The reaction was catalyzed by 8 units of alcohol dehydrogenase (*Thermoanaerobium brockii*, Sigma) at 43 °C for 1h and then maintained at 60 °C for 10 min. Labeled NADPH products were separated by HPLC and lyophilized as above.

The structures and stereochemistry of *S*-[4- ^2H] NADPH and *R*-[4- ^2H] NADPH were confirmed by one-dimensional ^1H -NMR spectroscopy (JNM-EX, 270 Hz, JEOL). The ^1H -NMR spectrum of non-labeled NADPH showed signals at δ 2.70 (dt, $J=1.8, 18.9$ Hz) for 4-*pro-R* hydrogen, and at δ 2.58 (dd, $J=2.7, 18.9$ Hz) for 4-*pro-S* hydrogen. *S*-[4- ^2H] NADPH showed a signal at δ 2.65 (t, $J=1.8$ Hz) for H-4, whereas a signal at δ 2.57 (d, $J=2.7$ Hz) was detected for 4-H of *R*-[4- ^2H] NADPH. These signals were in good accordance previously published data

(Mostad and Glasfeld, 1993). The ratios of *S*- and *R*-[4-²H] NADPH were calculated to be 83% and 85% based on the intensities of proton signals assigned to 4-pro *R* and 4-pro *S*, respectively.

Classification of rose-PAR and recombinant PAR by elucidating the enantio-selectivity toward *R*-[4-²H]-NADPH or *S*-[4-²H]-NADPH

To clarify the enantio-selectivity of both PARs, rose-PAR (46.8 µg) and recombinant PAR (53.4 µg) were used to catalyze the reaction of 40 µL 2.5 mM PAld in the presence of either *R*-[4-²H]-NADPH or *S*-[4-²H]-NADPH in 100 µL 100 mM potassium phosphate at 30 °C for 20 min or at 60 °C for 5 min, respectively. Ethyl decanoate (4 µL of 7.8 mM solution) was added as internal standard. The reaction solution was extracted 3 times with a mixture of 200 µL hexane-ethyl acetate (1:1 v/v). The combined organic layers were dried over sodium sulfate and subjected to GC-MS analysis.

The GC-MS analysis was conducted on a GCMS-QP5000 (Shimadzu) equipped with a SUPELCOWAX 10 column (30 mm × 0.25 mm × 0.25 µm). The injector temperature was 230 °C and the samples were injected in split-less injection mode. The oven temperature was set to 60 °C and maintained for 3 min, and the temperature increased to 180 °C at a heating rate of 40 °C min⁻¹. Finally, the temperature was increased to 240 °C at a heating rate of 10 °C min⁻¹ and the temperature hold for 3 min. Masses were recorded from *m/z* 76 to 400 with an electric potential of 1.25 kV. Identification of PAld and 2PE was based on a comparison of their MS spectra and retention times with those of authentic samples. Enantio-selectivities for chiral NADPHs were determined based on peak intensities at *m/z* 122 [*M*⁺] for 2PE and *m/z* 123 [*M*⁺] for [1-²H]-2PE.

Enantio-selectivity of the recombinant PAR toward acetophenone

To clarify an enantio-selectivity of recombinant PAR on the keto-carbonyl moieties, acetophenone was used as a model keto-carbonyl compound. The reaction of 10 mM acetophenone with 2.5 mM NADPH was catalyzed by recombinant PAR (53.4 µg) in 0.1 M potassium phosphate buffer at 30 °C for 60 min, and at 60 °C for 10 min, respectively. Reaction products were extracted as described above and subjected to GC-MS analysis. The GCMS-QP5000 (Shimadzu) was equipped with a

chiral InterCap CHIRAMIX column (30 mm × 0.25 mm × 0.25 μm), the oven temperature was set to 40 °C for 5 min, and then temperature was increased to 180 °C at a rate of 3 °C min⁻¹. Mass scan range was from *m/z* 70 to *m/z* 400 with an electronic potential of 1.25 kV. The volume of injection was 1 μL. The stereochemistry of 1-phenylethanol (1PE) was confirmed by authentic standards. The retention times of *S*-1PE and *R*-1PE were 36.0 and 36.3 min, respectively. Enantioselectivity was determined based on the ratio of *S*-/*R*-1PE.

Results

Isolation of a full length rose-PAR cDNA from *Rosa ×damascena*

To identify the full length cDNA based on partial amino acid sequences from rose-PAR, we partially purified the enzyme from petals of *R. ×damascena*. Predominant PAR activities were found in fractions 21 to 23 Fig. 1A. Although fraction 22 was not perfectly purified, this fraction showed the highest rose PAR activity. We excised proteins of fraction 22 from the SDS-PAGE gel, especially focused on the band detected at *ca* 35 kDa, based on the molecular masses of *Solanum lycopersicum* PAR1 and PAR2 previously reported (Tieman et al., 2007). The proteins were digested with trypsin prior to LC-MS/MS analysis of partial amino acid sequences of rose-PAR.

De novo sequence analyses of the protein band (designated as band 1 in Fig. 1B) resulted in 11 partial peptide sequences (106 amino acids). Three degenerate primers were designed for cDNA cloning based on the *de novo* sequences (Table 1). As a result of 3'-RACE amplification, sequence fragments of 465 bp were determined. A full-length cDNA was subsequently obtained using gene-specific primers (GSP) for 5'-RACE. The nucleotide sequence of this cDNA has an open reading frame of 966 bp that encodes a predicted protein of 322 amino acids comprising the 11 partial amino acid sequences derived from the partial purified protein of rose petals (Supplementary Fig. 1). Only one *PAR* cDNA was obtained from *R. ×damascena*. The nucleotide sequence designated as *recombinant PAR* is available from the DDBJ/EMBL databases under the accession number AB426519. The deduced amino acid sequence of recombinant PAR has 77% and 75% identity with *Solanum lycopersicum* PAR1 and PAR2, respectively. Phylogenetic analysis of the deduced protein sequence showed high similarity with aldehyde reductases, such as cinnamyl

alcohol dehydrogenases and cinnamyl CoA reductases from many plant species (Supplementary Fig. 2). The protein encoded by *PAR* cDNAs was closely related to a putative cinnamyl alcohol dehydrogenase from *Malus domestica* (90% identity) and *Prunus mume* (89% identity) (Mita et al., 2006). The recombinant PAR was also highly similar to two aldehyde reductases from *Solanum lycopersicum* (Tieman et al., 2007) (77% identity with *PAR1* and 75% identity with *PAR2*). The recombinant PAR has a calculated molecular mass of 35.4 kDa, which is in accordance with SDS-PAGE results of purified recombinant and rose-PAR enzymes (Fig. 1).

Functional characterization of rose-PAR and recombinant PAR

To confirm that the cloned cDNA encodes a functional enzyme, reaction products in the presence of NADPH and either rose-PAR or recombinant PAR were analyzed by GC-MS (Supplementary Scheme 1). No reaction products were detected in the absence of either PAR enzyme, whereas 2PE was detected as the sole product from PAld in the presence of either rose-PAR or recombinant PAR, indicating that both proteins exhibit the same functions (Fig. 2).

Changes in transcripts of rose-PAR

To further substantiate rose-*PAR*'s involvement in the biosynthesis of 2PE, expression of rose-*PAR* transcripts in petals, calyxes at stages 2, 4, and 6, and leaves were investigated by real time RT-PCR (Fig. 3). In *R. ×damascena* the transcripts of rose-*PAR* were higher in petals than in calyxes and leaves. In the petals the transcripts of rose-*PAR* were peaking at stage 4. There was no obvious difference in the expression level of rose-*PAR* among calyxes throughout the unfurling process.

Coenzyme specificity and catalytic activity of recombinant PAR and rose-PAR

Recombinant PAR efficiently converted PAld to 2PE in the presence of NADPH, whereas only a trace amount of 2PE was synthesized in the presence of NADH (Fig. 4A). Similar results were obtained with rose-PAR. Thus, PAR is a NADPH-preferring reductase.

Furthermore, the biosynthetic pathway proposed by Sakai et al. (2007) for production of 2PE from PAld was catalyzed by PAR but the inverse reaction would

be hypothetically catalyzed by an alcohol dehydrogenase (ADH). The recombinant PAR enzyme has around 10 times higher reductase activity than ADH activity. The rose-PAR has high reductase activity with only residual ADH activity, indicating that both recombinant PAR and rose-PAR predominantly catalyze the conversion of PAld to 2PE (Fig. 4B).

Substrate specificity of recombinant PAR and rose-PAR

To understand the function of an enzyme in its metabolic pathway, enzymes and their substrates must be characterized (Fridman et al., 2005). To elucidate the substrate specificity of recombinant PAR and rose-PAR more in detail, 11 different substrates with either aldehyde or keto moieties were tested (Table 2). Catalytic efficiency of the recombinant PAR with (*S*)-(-)-citronellal was the highest among all of the selected substrates, including a 3-fold increase over PAld. Hexylaldehyde also had a higher turn over rate (1.9 fold) compared to PAld. Even though, the specific activity of the rose-PAR (10.1 mU mg⁻¹) was much higher (10-fold) than the recombinant PAR (0.7 mU mg⁻¹), both PAR enzymes showed activity with all of the selected volatile compounds. The catalytic efficiencies of the rose-PAR and the recombinant PAR were high using PAld, (*S*)-(-)-citronellal and hexylaldehyde as substrates. These enzymes showed moderate catalytic activities with the aldehydes: (*R*)-(-)-citronellal (96.9, 46.6), 3-phenylpropionaldehyde (63.6, 59.2), benzaldehyde (47.3, 54.0), *trans*-cinnamaldehyde (40.3, 14.8), 2-phenylpropionaldehyde (39.5, 19.5), and citral (39.5, 53.9). Low activities were observed for the transformations of acetophenone (28.7, 7.0) and methyl butylketone (19.0, 7.5). It can thus be inferred that the catalytic efficiency of PAR is higher with aldehydes than with compounds of the ketocarbonyl group. The catalytic activity of the recombinant PAR was 3-fold higher with (*S*)-citronellal (311.2) than with its (*R*)-isomer (96.9) and the activity of the rose-PAR was 2 times higher with (*S*)-citronellal (78.4) than with its (*R*)-isomer (46.6).

Enantio-selective reduction of recombinant PAR

In our assay, both isomers of NADPH were labeled with mono-deuterium. Incubation of the recombinant PAR and the rose-PAR with *R*-[4-²H] NADPH or *S*-[4-²H] NADPH resulted in 96.6% and 72.6% of [²H]-2PE respectively, whereas in the

315 presence of *R*-[4-²H] NADPH, the [²H]-2PE production was lower (12.0% with the
316 recombinant PAR and 17.9% with the rose-PAR) (Fig. 5). Thus, almost 90% of the
317 PAld was converted to [²H]-2PE when *S*-[4-²H] NADPH was used. Even in the case
318 of the rose-PAR, the deuterium incorporation of *S*-[4-²H] NADPH was 83%. Hence,
319 both PAR enzymes preferred *S*-[4-²H] NADPH over *R*-[4-²H] NADPH. Furthermore,
320 the reduction of PAld with *S*-[4-²H] NADPH and the recombinant PAR was more
321 efficient (96.6% production of [²H]-2PE) than with the rose-PAR (72.6% production
322 of [²H]-2PE).

323 **Stereo-selectivity of recombinant PAR**

324 To investigate the stereo-selectivity of the recombinant PAR for the keto-
325 carbonyl group to yield to its corresponding secondary alcohol, acetophenone was
326 employed as model substrate (Fig. 6). Reaction mixture of acetophenone and
327 recombinant PAR yielded *S*-1PE in the presence of NADPH. Due to the low catalytic
328 activity of the rose-PAR with the substrate acetophenone the enantio-selectivity could
329 not be determination.

330 **Discussion**

331 We have isolated a full-length PAR cDNA from *R. ×damascena*, and have
332 functionally characterized both recombinant PAR and rose-PAR. Even though a
333 protein-protein BLAST (blastp) search revealed that the obtained PAR is more similar
334 to the cinnamyl alcohol dehydrogenase from *Malus domestica* (90% identity) than to
335 the phenyl acetaldehyde reductases from *Solanum lycopersicum* (77% and 75%
336 identity), functional characterization clearly demonstrated that the PARs catalyzes the
337 transformation of PAld to 2PE. Frequently, functional enzyme annotations based on
338 sequence similarities prove to be incorrect (Fridman et al., 2005). For example, many
339 *Arabidopsis* genes annotated as putative cinnamyl alcohol dehydrogenases actually
340 encode enzymes with highly variable substrate specificities (Kim et al., 2004).

341 The GC-MS-validated functional analysis of both rose-PAR and recombinant
342 PAR confirmed that the PARs catalyze the conversion of PAld to 2PE. This study
343 revealed for the first time that rose-PAR can contribute to the production of important
344 scent molecules on molecular level. Furthermore, we investigated changes in
345 transcripts of rose-*PAR* by real time RT-PCR. Rose-*PAR* transcripts were higher in

petals than in calyxes and leaves. The highest transcripts have been observed at stage 4, suggesting a correlation to the maximum emission of 2PE at stage 4 of *R. ×damascena* as already reported (Oka et al., 1999). Other rose scent-related genes exhibited the highest transcripts at the same unfurling stage, where the emission of volatile compounds was the highest (Guterman et al., 2002; Lavid et al., 2002; Farhi et al., 2010). Both rose-PAR and recombinant PAR preferred NADPH over NADH as coenzyme (Fig. 4A), which differs from what was observed for the PAR isolated from *R. 'Hoh-Jun'* (Sakai et al., 2007). Sakai et al. had reported that both NADPH and NADH could serve as cofactors for rose-PAR. Although further research is needed, this discrepancy might be due to the incomplete purity of rose-PARs in the *R. 'Hoh-Jun'* assays. For example, in this study the PAR enzyme was separated from other proteins with a 30% to 70% ammonium sulfate cut, but previously with 20% to 50% (Sakai et al., 2007). In this case, an enzyme could have been co-precipitated with PAR which is eliminated by the higher salt concentration in the first cut. Alternatively, rose cultivars may produce similar enzymes with differing substrate and co-enzyme binding affinities. This would, in fact, be expected since different cultivars produce different scents. For example, glucose-6-phosphate dehydrogenases (G6PDHs) catalyzed the oxidation of glucose-6-phosphate to 6-phosphogluconolactone concomitant with reducing NADP to NADPH, and an elevated level of cytosolic glucose-6-phosphate dehydrogenases (G6PDHs) was not a consequence of phosphate sequestration, but rather dependent on the presence of metabolizable sugars (Hauschild and Schaewen; 2003). Furthermore, both PARs preferably catalyzed the reaction from PAld to 2PE (Fig. 4B), indicating that the genuine *PAR* had been cloned into *E. coli*. Consistent with our results, most cinnamyl alcohol dehydrogenase/reductase enzymes, including PAR1 and PAR2 from *Solanum lycopersicum*, also prefer NADPH as co-substrate (Tieman et al., 2007).

The recombinant PAR had a substrate-utilization profile similar to the rose-PAR (Table 2). Both PARs favored aldehyde substrates over compounds with keto-carbonyl moieties. Moreover, both PARs had higher catalytic activities on the (*S*)-citronellal enantiomer, indicating that PAR activities are affected by chirality at the C-6 position. The rose-PAR and the recombinant PAR differed somewhat in substrate affinity. For instance, (*S*)-(-)-citronellal was the best substrate among a variety of volatile compounds for the recombinant PAR, but for the native rose-PAR, PAld was the best substrate. For the PAR two sugar modification motifs, NTSA in No. 201-204

and NASF in No. 279-282 were predicted based on the Motif search by GENETYX as shown in Supplementary Fig. 1. It is generally known that proteins obtained by *E. coli* lack in post-translational modifications. Although the sugar analysis was not performed against rose-PAR, the lack in the sugar motives in recombinant PAR probably is one of the reasons for the differences in the substrate specificity. It has been already reported that sugar modification could affect relative enzyme functions (Hauschild and Schaewen, 2003). In addition, using surfactants or CA kit (TaKaRa Co. Ltd. Japan) could not overcome the different catalytic activities of rose-PAR and recombinant PAR enzymes (data not shown).

Our group previously detected various volatile scent compounds emitted from *R. ×damascena* throughout the unfurling process (Oka et al., 1999). Several reductases as well as the rose-PAR may be involved in the emission of other alcoholic volatile compounds such as (*S*)-(-)-citronellol and geraniol. It might be reasonable to elucidate if rose-PAR plays an essential part for the production of several main rose scents (Table 2). The enantio-selectivity toward *S*-[4-²H] NADPH gives the basic aspects on the biosynthesis of 2PE and other primary alcoholic plant volatiles from the corresponding aldehydes. It may also explain the selectivity of PAR between two chiral aldehydes such as (*S*)- and (*R*)-citronellal. Further research will afford the evidence to explain the direction of approach for *S*-[4-²H] NADPH and the substrate in the active domain of the enzyme.

The rose-PAR and recombinant PAR exhibited moderate activities toward keto-carbonyl compounds, and the latter yielded *S*-1PE from acetophenone (Table 2, Fig. 6). These results may be illustrating to find the high enantio-selectivity for production of chiral secondary alcohols by modifying the recombinant PAR. Also *S*-selectivity of rose-PAR toward acetophenone must be important to elucidate the role in the keto-reduction in rose flowers. As neither acetophenone nor 1PE were detected as volatile compounds emitted from *R. ×damascena*, this rose may not have the biosynthetic systems of acetophenone. As one of the precursors of damascenone, an important volatile compound found in the essential oil, we have reported (Suzuki et al., 2002) the identification of glycosidic (3*R*, 9*R*)- and (3*R*, 9*S*)-megastigm-6, 7-dien-3, 5, 9- triol in the flowers of *R. ×damascena*. In the case of the production of these compounds, a progenitor of the aglycon parts must be derived from (3*R*)-megastigm-6, 7-dien-9-one-3, 5-diol by the action of 9-keto-reductase. As the ratio at the 9-position

of glycosidic (3*R*, 9*R*)- and (3*R*, 9*S*)-megastigm-6, 7-dien 3, 5, 9- triol was 4-10 /1 for *R/S*, rose-PAR is not involved in the reduction of the 9-keto-carbonyl group.

Furthermore, the substrate specificities and relative activities of rose-PARs from *R. ×damascena* and *R. 'Hoh-Jun'* are generally similar (Sakai et al., 2007). For instance, both native PARs had higher activities with aldehydes than with substrates with ketocarbonyl moieties, and PAld was the best substrate for both native PARs. However, *R. ×damascena* rose-PAR catalyzes reactions with a wider range of substrates than *R. 'Hoh-Jun'* rose-PAR, which did not show any activity with benzaldehyde, *trans*-cinnamaldehyde, acetophenone or methyl butylketone.

A commonly used sequence-based classification of alcohol dehydrogenases defines three super-families which are differentiated, amongst other features, by the molecular size of the protein chain: short-chain dehydrogenase/reductases (SDR; ~250 amino acids) (Jornvall et al., 1995), medium-chain dehydrogenase/reductases (MDR; ~350 amino acids per subunit) (Persson et al., 1994), and long-chain dehydrogenases (LDR; ~360-550 amino acids) (Persson et al., 1991). An increasing number of oxidoreductases not related to any of these superfamilies have been identified as members of the aldo-keto reductase (AKR) superfamily (Bohren et al., 1989). Only the AKRs are monomeric proteins among these four superfamilies, and are about 320 amino acid residues in size.

The SDRs and LDRs utilize *pro-S* hydrogen of NADPH, whereas the MDRs and AKRs utilize *pro-R* hydrogen (Costanzo et al., 2009). Thus, as a potential discriminator for classification, the purified enzyme preparations were assayed with *S*-, and *R*-[4-²H]-NADPH to convert PAld or acetophenone. Both recombinant PAR and rose-PAR preferred *S*-[4-²H] NADPH over *R*-[4-²H] NADPH (Fig. 5), suggesting that both PARs are SDRs or LDRs rather than MDRs and AKRs. Structurally, SDR functional sites contain a YXX(S)K motif, whereas the AKR cofactor-binding pocket has four strictly-conserved residues (D50, Y55, K84 and H117). PAR contains a YVLSK sequence at residues 60 to 64, and no AKR cofactor-binding pocket motif (Supplementary Fig. 1). This suggests that recombinant PAR and rose-PAR may be placed in the SDR protein super-family.

Acknowledgements

We wish to thank Dr. Vipin Kumar Deo for critically reading the manuscript.

- Albertazzi E, Cardillo R, Servi S, Zucchi G. Biogenesis of 2-phenylethanol and 2-phenylethylacetate important aroma components. *Biotechnol Lett* 1994;16:491-6.
- Bauer K, Garbe D, Surburg H. Common fragrance and flavor materials preparations, properties and uses. Weinheim: Wiley-VCH, 2001.
- Berrah G, Konezka WA. Selective and reversible inhibition of the synthesis of bacterial deoxyribonucleic acid by phenethyl alcohol. *J Bacteriol* 1962;83:738-44.
- Bohren KM, Bullock B, Wermuth B, Gabbay KH. The aldo-keto reductase superfamily. *J Biol Chem* 1989;264:9547-51.
- Bugorskii PS, Zaprometov MN. Biosynthesis of beta-phenylethanol in rose petals. *Biokhimiia* 1978;43:2038-42.
- Clark GS. Phenylethyl alcohol. *Perfum Flavor* 1990;15:37-44.
- Costanzo LD, Penning TM, Christianson DW. Aldo-keto reductases in which the conserved catalytic histidine is substituted. *Chem-Biol Inter* 2009;178:127-33.
- Edman P. Method for determination of the amino acid sequence in peptides. *Acta Chem Scand* 1950;4:283-93.
- Fabre CE, Blanc PJ, Goma G. 2-Phenylethyl alcohol: An aroma profile. *Perfum Flavor* 1998;23:43-5.
- Farhi M, Lavie O, Masci T, Hendel-Rahmanim K, Weiss D, Abeliovich H, Vainstein A. Identification of rose phenylacetaldehyde synthase by functional complementation in yeast. *Plant Mol Biol* 2010;72:235-45.
- Fleischmann P, Watanabe N, Winterhalter P. Enzymatic carotenoid cleavage in star fruit (*Averrhoa carambola*). *Phytochemistry* 2003;63:131-7.
- Fridman E, Pichersky E. Metabolomics, genomics, proteomics, and the identification of enzymes and their substrates and products. *Curr Opin Plant Biol* 2005;8:242-8.
- Guterman I, Shalit M, Menda N, Piastun 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, Weiss D. Rose scent: genomics approach to discovering novel floral fragrance-related genes. *Plant Cell* 2002;14:2325-38.
- Hauschild R, Schaewen AV. Differential regulation of glucose-6-phosphate dehydrogenase isoenzyme activities in potato. *Plant Physiol* 2003;133:47-62.
- Hayashi S, Yagi K, Ishikawa T, Kawasaki M, Asai T, Picone J, Turnbull C, Hiratake J, Sakata K, Takada M, Ogawa K, Watanabe N. Emission of 2-phenylethanol from its β -D-glucopyranoside and the biogenesis of these compounds from [$^2\text{H}_8$] L-phenylalanine in rose flowers. *Tetrahedron* 2004;60:7005-13.
- Jollivet N, Bézenger MC, Vayssier Y, Belin JM. Production of volatile compounds in liquid cultures by six strains of coryneform bacteria. *Appl Microbiol Biotechnol* 1992;36:790-94.
- Jornvall H, Persson B, Krook M, Atrian S, Gonzalez-Duarte R, Jeffery J, Ghosh D. Short-chain dehydrogenases/reductases (SDR). *Biochemistry* 1995;34:6003-13.
- Kaminaga Y, Schnepf J, Peel G, Kish CM, Ben-Nissan G, Weiss D, Orlova I, Lavie O, Rhodes D, Wood K, Porterfield DM, Cooper AJL, Schloss JV, Pichersky E, Vainstein A, Dudareva N. Plant phenylacetaldehyde synthase is a bifunctional homotetrameric enzyme that catalyzes phenylalanine decarboxylation and oxidation. *J Biol Chem* 2006;281:23357-66.
- Larroy C, Fernandez MR, Gonzalez E, Pares X, Biosca JA. Characterization of the *Saccharomyces cerevisiae* YMR318C (*ADH6*) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction. *Biochem J* 2002;361:163-72.
- Lavid N, Wang J, Shalit M, Guterman I, Bar E, Beuerle T, Menda N, Shafir S, Zamir D, Adam Z, Vainstein A, Weiss D, Pichersky E, Lewinsohn E. O-Methyltransferases involved in the biosynthesis of volatile phenolic derivatives in rose petals. *Plant Physiol* 2002;129:1899-907.
- Mabberley DJ. Mabberley's Plant-Book. Cambridge: Cambridge University Press, 2008.
- McCracken JA, Wang L, Kohen A. Synthesis of R and S tritiated reduced β -nicotinamide adenine dinucleotide 2' phosphate. *Anal Biochem* 2004;324:131-6.
- Mita S, Nagai Y, Asai T. Isolation of cDNA clones corresponding to genes differentially expressed in pericarp of mume (*Prunus mume*) in response to ripening, ethylene and wounding signals. *Physiol Plant* 2006;128:531-45.
- Mostad SB, Glasfeld A. Using high field NMR to determine dehydrogenase stereospecificity with respect to NADH. *J Chem Educ* 1993;70:504-6.
- Niall HD. Automated Edman degradation: the protein sequencer. *Methods Enzymol* 1973;27:942-1010.

Oka N, Ohishi H, Hatano T, Hornberger M, Sakata K, Watanabe N. Aroma evolution during flower opening in *Rosa damascena* Mill. *Z Naturforsch* 1999;54c:889-95.

Okada T, Mikage M, Sekita S. Molecular characterization of the phenylalanine ammonia-lyase from *Ephedra sinica*. *Biol Pharm Bull* 2008;31:2194-9.

Persson B, Jeffery J, Jörnvall H. Different segment similarities in long-chain dehydrogenases. *Biochem Biophys Res Commun* 1991;177:218-23.

Persson B, Zigler JJ, Jörnvall H. A super-family of medium-chain dehydrogenases/reductases (MDR). *Eur J Biochem* 1994;226:15-22.

Persson B, Kallberg Y, Oppermann U, Jörnvall H. Coenzyme-based functional assignments of short-chain dehydrogenases/reductases (SDRs). *Chem Biol Interact* 2003;143-144:271-8.

Pichersky E, Gershenzon J. The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Curr Opin Plant Biol* 2002;5:237-43.

Rodopulo AK, Lyudnikova TA, Bezzubov AA. Effect of yeast cultivation conditions on the biosynthesis and accumulation of aromatic substances. *Appl Biochem Microbiol* 1985;21:332-6.

Rusanov K, Kovacheva N, Vosman B, Zhang L, Rajapakse S, Atanassov A, Atanassov I. Microsatellite analysis of *Rosa damascena* Mill. accessions reveals genetic similarity between genotypes used for rose oil production and old Damask rose varieties. *Theor Appl Genet* 2005;111:804-9.

Sakai M, Hirata H, Sayama H, Sekiguchi K, Itano H, Asai T, Dohra H, Hara M, Watanabe N. Production of 2-phenylethanol in roses as the dominant floral scent compounds from L-phenylalanine by two key enzymes, a PLP-dependent decarboxylase and a phenylacetaldehyde reductase. *Biosci Biotechnol Biochem* 2007;71:2408-19.

Serra S, Fuganti C, Brenna E. Biocatalytic preparation of natural flavours and fragrances. *Trends Biotechnol* 2005;23:193-8.

Suzuiki M, Matsumoto S, Mizoguchi M, Hirata S, Takagi K, Hashimoto I, Yamano Y, Ito M, Fleischmann P, Winterhalter P, Morita T, Watanabe N. Identification of (3S, 9R)- and (3S, 9S)-megastigma-6,7-dien-3,5,9-triol 9-O-β-D-glucopyranosides as damascenone progenitors in the flowers of *Rosa damascena* Mill. *Biosci Biotechnol Biochem* 2002;66:2692-7.

Tieman D, Taylor M, Schauer N, Fernie AR, Hanson AD, Klee HJ. Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. *Proc Natl Acad Sci* 2006;103:8287-92.

Tieman DM, Loucas HM, Kim JY, Clark DG, Klee HJ. Tomato phenylacetaldehyde reductases catalyze the last step in the synthesis of the aroma volatile 2-phenylethanol. *Phytochemistry* 2007;68:2660-9.

Watanabe S, Hayashi K, Yagi K, Asai T, MacTavish H, Picone J, Turnbull C, Watanabe N. Biogenesis of 2-phenylethanol in rose flowers: incorporation of [²H₈] 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* 2002;66:943-7.

Zhang L, Kudo T, Takaya N, Shoun H. The B' helix determines cytochrome P450 nor specificity for the electron donors NADH and NADPH. *J Biol Chem* 2002;277:33842-7.

Tables

Table 1. Peptide fragments of rose-PAR from *Rosa ×damascena*

MH ⁺	m/z	Charge	Sequence	Degenerate primer
839.4	420.2	2+	YCLVER	
908.5	454.8	2+	LWYVLSK	
955.5	478.2	2+	AELDPAVK	
974.5	487.7	2+	YHDVTDPK	No. 1
1004.5	502.8	2+	TLAEDAAWK	No. 2
1076.6	538.8	2+	ETLESLKEK	
1167.7	584.3	2+	TEHLLALDGAK	
1192.6	596.8	2+	GTLNVLNSCSK	
1198.6	400.2	3+	ASVRNPNDPTK	

1512.8	756.9	2+	TYPNASF	
1868.9	623.6	3+	DVANAHVQAFELPSASGR	No. 3

Table 2. Relative activities of the recombinant PAR and rose-PAR from *Rosa ×damascena* with selected substrates. Enzymatic activities with phenylacetaldehyde were set as 100%. Data present the mean values $\pm$ standard error from triplicate experiments.

Substrate	Recombinant PAR relative activity (%)	Rose- PAR relative activity (%)
Phenylacetaldehyde	100.0 $\pm$ 4.9	100.0 $\pm$ 1.4
(S)-(-)-Citronellal	311.2 $\pm$ 8.7	78.4 $\pm$ 2.3
Hexylaldehyde	186.0 $\pm$ 6.5	66.4 $\pm$ 1.7
(R)-(+)-Citronellal	96.9 $\pm$ 4.9	46.6 $\pm$ 1.2
3-Phenylpropionaldehyde	63.6 $\pm$ 5.7	59.2 $\pm$ 0.9
Benzaldehyde	47.3 $\pm$ 4.4	54.0 $\pm$ 2.8
trans-Cinnamaldehyde	40.3 $\pm$ 4.2	14.8 $\pm$ 0.9
2-Phenylpropionaldehyde	39.5 $\pm$ 6.2	19.5 $\pm$ 1.2
Citral	39.5 $\pm$ 6.6	53.9 $\pm$ 2.8
Acetophenone	28.7 $\pm$ 3.9	7.0 $\pm$ 1.0
Methyl butylketone	19.0 $\pm$ 1.9	7.5 $\pm$ 2.4

Fig. 1. Isolation and identification of rose-PAR by gel filtration chromatography and SDS-PAGE. (A) PAR activity in gel filtration chromatographic fractions assayed by absorbance changes of NADPH. (B) SDS-PAGE of chromatographic fractions 21-23. The 35 kDa protein occurs in fraction 22 which had the highest enzymatic activity.

Fig. 2. Conversion from PAld to 2PE by the recombinant PAR expressed in *E. coli* and rose-PAR. Products were separated by GC. The control assay was carried out without enzyme.

Fig. 3. Relative transcripts expression levels of *PAR* in different rose tissues at different stages. Values represented the ratio of *PAR* transcripts (from 50 ng RNA) to *TUB*. Data shown represent the mean values $\pm$ standard deviation from triplicate experiments.

Fig. 4. Coenzyme preference and direction of reactions catalyzed by PARs. A, Coenzyme preference. PAR activity was assayed in the presence of 1mM NADPH or NADH. The activity of the NADPH sample (1.5 mmol mg⁻¹ protein h⁻¹) is regarded as 100%. B, Reaction direction of PAR. Oxidative activity (ADH activity) for the

production of 2PE in the presence of NADP^+ was measured by GC-MS. The reaction mixture contained 1mM 2PE, 1 mM NADP^+ and 30 μl of the main PAR fraction. PAR activity ($1.3 \text{ mmol mg}^{-1} \text{ protein h}^{-1}$) was set as 100%. All data shown represent the mean $\pm$ standard error from triplicate experiments.

Fig. 5. The selectivities of both recombinant and rose-derived PARs for the conversion of PAld to 2PE in the presence of NADPH. Both NADPH enantiomers were labeled with ^2H . The total amount of 2PE and $[^2\text{H}]$ -2PE is set as 100%.

Fig. 6. GC analysis of the reaction products of acetophenone catalyzed by the recombinant PAR. A: total ion traces of authentic samples; B: reaction mixture.