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

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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 [4S-2H] NADPH was preferentially used over the [4R-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.
Introduction

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

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

**Molecular cloning of PAR from Rosa ×damascena Mill.**
Total RNA was isolated from the flower petals of *R. × damascena* with a RNasy 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 DH5α 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 μg 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 μL 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 (ε₃₄₀= 6.2 mM⁻¹ cm⁻¹,
Ultrospec 3000, Pharmacia Biotech) (Larroy et al., 2002). The reaction mixture (200
µL) (100 mM potassium phosphate (pH 7.0), recombinant enzyme (6.8 µg)/rose-
PAR (8.0 µg), 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 µL 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 NH₄HCO₃ 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 [²H₈] 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 ᵈ¹-NMR spectroscopy (JNM-EX, 270 Hz, JEOL). The ᵈ¹-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 1 µL
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 \textit{PAR} cDNAs was closely related to a putative cinnamyl alcohol dehydrogenase from \textit{Malus domestica} (90\% identity) and \textit{Prunus mume} (89\% identity) (Mita et al., 2006). The recombinant PAR was also highly similar to two aldehyde reductases from \textit{Solanum lycopersicum} (Tieman et al., 2007) (77\% identity with \textit{PAR1} and 75\% identity with \textit{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 \textit{R. \times 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-\(^2\)H] NADPH or \(S\)-[4-\(^2\)H] NADPH resulted in 96.6% and 72.6% of [\(^2\)H]-2PE respectively, whereas in the
presence of $R$-[4-$^2$H] NADPH, the [$^2$H]-2PE production was lower (12.0% with the recombinant PAR and 17.9% with the rose-PAR) (Fig. 5). Thus, almost 90% of the PAld was converted to [$^2$H]-2PE when $S$-[4-$^2$H] NADPH was used. Even in the case of the rose-PAR, the deuterium incorporation of $S$-[4-$^2$H] NADPH was 83%. Hence, both PAR enzymes preferred $S$-[4-$^2$H] NADPH over $R$-[4-$^2$H] NADPH. Furthermore, the reduction of PAld with $S$-[4-$^2$H] NADPH and the recombinant PAR was more efficient (96.6% production of [$^2$H]-2PE) than with the rose-PAR (72.6% production of [$^2$H]-2PE).

**Stereo-selectivity of recombinant PAR**

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

**Discussion**

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

The GC-MS-validated functional analysis of both rose-PAR and recombinant PAR confirmed that the PARs catalyze the conversion of PAld to 2PE. This study revealed for the first time that rose-PAR can contribute to the production of important scent molecules on molecular level. Furthermore, we investigated changes in 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-phosphogluonolactone 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-carobonyl compounds, and the latter yielded S-1PE from acetophenone (Table 2, Fig. 6). These results may be illustrating to find the high enantio-selectity for production of chiral secondary alcohols by modifying the recombinant PAR. Also S-selectivity of rose-PAR toward acetophonone 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 (3R, 9R)- and (3R, 9S)-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 (3R)-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 (3R, 9R)- and (3R, 9S)-megastigm-6, 7-dien 3, 5, 9-triol was 4-10 /1 for 413  
R/S, rose-PAR is not involved in the reduction of the 9-keto-carbonyl group. 414  
Furthermore, the substrate specificities and relative activities of rose-PARs 415  
from R. ×damascena and R. ‘Hoh-Jun’ are generally similar (Sakai et al., 2007). For 416  
instance, both native PARs had higher activities with aldehydes than with substrates 417  
with ketocarbonyl moieties, and PAld was the best substrate for both native PARs. 418  
However, R. ×damascena rose-PAR catalyzes reactions with a wider range of 419  
substrates than R. ‘Hoh-Jun’ rose-PAR, which did not show any activity with 420  
benzaldehyde, trans-cinnamaldehyde, acetophenone or methyl butylketone. 421  
A commonly used sequence-based classification of alcohol dehydrogenases 422  
defines three super-families which are differentiated, amongst other features, by the 423  
molecular size of the protein chain: short-chain dehydrogenase/reductases (SDR; 424  
~250 amino acids) (Jornvall et al., 1995), medium-chain dehydrogenase/reductases 425  
(MDR; ~350 amino acids per subunit) (Persson et al., 1994), and long-chain 426  
dehydrogenases (LDR; ~360-550 amino acids) (Persson et al., 1991). An increasing 427  
number of oxidoreductases not related to any of these superfamilies have been 428  
identified as members of the aldo-keto reductase (AKR) superfamily (Bohren et al., 429  
1989). Only the AKRs are monomeric proteins among these four superfamilies, and 430  
are about 320 amino acid residues in size. 431  
The SDRs and LDRs utilize pro-S hydrogen of NADPH, whereas the MDRs 432  
and AKRs utilize pro-R hydrogen (Costanzo et al., 2009). Thus, as a potential 433  
discriminator for classification, the purified enzyme preparations were assayed with 434  
S-, and R-[4-2H]-NADPH to convert PAld or acetophenone. Both recombinant PAR 435  
and rose-PAR preferred S-[4-2H] NADPH over R-[4-2H] NADPH (Fig. 5), suggesting 436  
that both PARs are SDRs or LDRs rather than MDRs and AKRs. Structurally, SDR 437  
functional sites contain a YXX(S)K motif, whereas the AKR cofactor-binding pocket 438  
has four strictly-conserved residues (D50, Y55, K84 and H117). PAR contains a 439  
YVLSK sequence at residues 60 to 64, and no AKR cofactor-binding pocket motif 440  
(Supplementary Fig. 1). This suggests that recombinant PAR and rose-PAR may be 441  
placed in the SDR protein super-family. 442  

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References


Mostad SB, Glasfeld A. Using high field NMR to determine dehydrogenase stereospecificity with respect to NADH. J Chem Educ 1993;70:504-6.


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

<table>
<thead>
<tr>
<th>MH⁺</th>
<th>m/z</th>
<th>Charge</th>
<th>Sequence</th>
<th>Degenerate primer</th>
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<tr>
<td>839.4</td>
<td>420.2</td>
<td>2+</td>
<td>YCLVER</td>
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</tr>
<tr>
<td>908.5</td>
<td>454.8</td>
<td>2+</td>
<td>LWYVLSK</td>
<td></td>
</tr>
<tr>
<td>955.5</td>
<td>478.2</td>
<td>2+</td>
<td>AELLPDAVK</td>
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</tr>
<tr>
<td>974.5</td>
<td>487.7</td>
<td>2+</td>
<td>YHDVTPAVK</td>
<td></td>
</tr>
<tr>
<td>1004.5</td>
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<td>2+</td>
<td>TLAEADAWK</td>
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<tr>
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<td>ETLESLKEK</td>
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<td>1192.6</td>
<td>596.8</td>
<td>2+</td>
<td>GTLNVLNCSK</td>
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<td>1198.6</td>
<td>400.2</td>
<td>3+</td>
<td>ASVRNPNPDPTK</td>
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</table>

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 ± standard error from triplicate experiments.

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

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 ± 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 1 mM 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 mmol mg⁻¹ protein h⁻¹) was set as 100%. All data shown represent the mean ± 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 [²H]-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.
A

![Graph A](image)

Recombinant PAR
Rose-PAR

B

![Graph B](image)

Relative activity (%) vs NADPH and NADH
PAR activity vs ADH activity

[Graph Details]

583

Recombinant PAR
Rose-PAR

[Graph Details 2]

584

A

![Graph C](image)

(R)-1PE
Acetophenone
(S)-1PE
Standards

B

![Graph D](image)

Relative ion intensity vs
30 35 35 t_r (min)

Sample