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Functional characterization of aromatic amino acid aminotransferase involved in 2-phenylethanol biosynthesis in isolated rose petal protoplasts

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ABSTRACT

In rose flowers, 2-phenylethanol (2PE) is biosynthesized from L-phenylalanine (L-Phe) *via* phenylacetaldehyde (PAld) by the actions of two enzymes, pyridoxal-5′-phosphate (PLP)-dependent aromatic amino acid decarboxylase (AADC) and phenylacetaldehyde reductase (PAR). We here report that *Rosa* 'Yves Piaget' aromatic amino acid aminotransferase produced phenylpyruvic acid (PPA) from L-Phe in isolated petal protoplasts. We have cloned three full length cDNAs (*RyAAAT1-3*) of aromatic amino acid aminotransferase families based on rose EST database and homology regions. The RyAAATs enzymes were heterogeneously expressed in *Escherichia coli* and characterized biochemically. The recombinant RyAAAT3 showed the highest activity toward L-Phe in comparison with L-tryptophan, L-tyrosine, D-Phe, glycine, and L-alanine, and showed 9.7-fold higher activity with L-Phe rather than PPA as a substrate. RyAAAT3 had an optimal activity at pH 9 and at 45–55 °C with α-ketoglutaric acid, and was found to be a PLP dependent enzyme based on the inhibition test using Carbidopa, an inhibitor of PLP-dependent enzymes. The transcript of *RyAAAT3* was expressed in flowers as well as other organs of *R.* 'Yves Piaget'. RNAi suppression of *RyAAAT3* decreased 2PE production, revealing the involvement of *RyAAAT3* in 2PE biosynthesis in rose protoplasts and indicating that rose protoplasts have potentially two different 2PE biosynthetic pathways, the AADC route and the new route *via* PPA from L-Phe.

Introduction

2-Phenylethanol (2PE) is one of the prominent scent compounds produced by Damask roses (Hayashi et al., 2004; Sakai et al., 2007; Yang et al., 2009), and in various fruits such as strawberry, tomato and grape varieties (Aubert et al., 2005). 2PE and phenylacetaldehyde (PAld) contribute toward characteristic flavors in wine and cheese (Marilley and Casey, 2004) producing a pleasantly sweet, flowery note at low concentrations, while PAld is nauseating and unpleasant at high levels (Tadmor et al., 2002). The world's annual production of 2PE is estimated to be approximately 10,000 tons in 2010 (Schwab et al., 2008; Hua and Xu, 2011).

2PE is biosynthesized from L-phenylalanine (L-Phe) with pyridoxal-5′-phosphate (PLP)-dependent aromatic amino acid decarboxylases (AADC) and phenylacetaldehyde reductases (PAR) in *planta* (Fig. 1A) (Sakai et al., 2007). AADC transformed L-Phe to PAld *via* the Schiff base, which was formed by a reaction between the amino group of L-Phe and a formyl group of PLP. PAld was also synthesized by plant PAld synthase (PAAS), a member of the AADC family, in *Petunia hybrida* (Kaminaga et al., 2006) and by AADC in *Solanum lycopersicum* (Tieman et al., 2006) and *Arabidopsis* (Gutensohn et al., 2011).

PAld is converted to 2PE by the action of PAR (Tieman et al., 2007; Chen et al., 2011). Thus, 2PE is synthesized from L-Phe *via* PAld by the action of both enzymes, AADC and PAR in plants.

Microorganisms biosynthesize 2PE from L-Phe *via* phenylpyruvic acid (PPA), called 'Ehrlich pathway' (Ehrlich, 1907), while there is no report about the Ehrlich pathway in *planta* so far. In microorganisms, the amino acid metabolism has been studied in detail, and

Abbreviations: 2PE, 2-phenylethanol; AAAT, aromatic amino acid aminotransferase; AADC, aromatic amino acid decarboxylase; AspAT, aspartate aminotransferase; AlaAT, alanine aminotransferase; *E. coli*, *Escherichia coli*; PAld, phenylacetaldehyde; PAR, phenylacetaldehyde reductase; L-Phe, L-phenylalanine; PheAT, phenylalanine aminotransferase; PLP, pyridoxal-5′-phosphate; PPA, phenylpyruvic acid; TrpAT, tryptophan aminotransferase; TyrAT, tyrosine aminotransferase.

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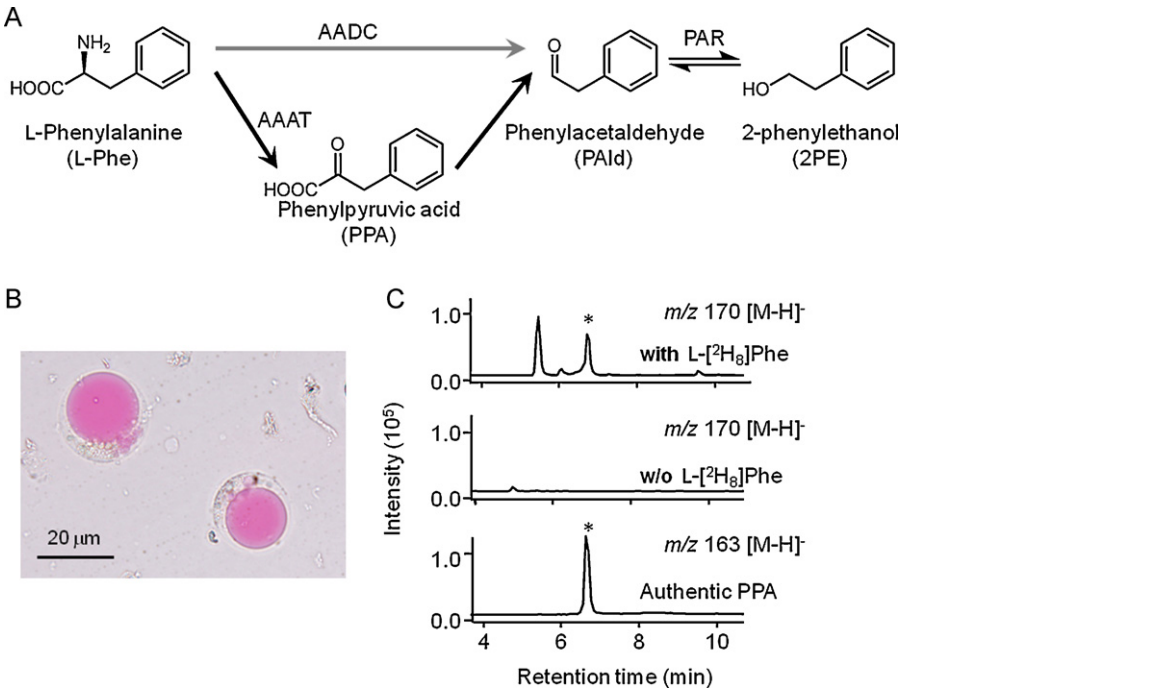

Fig. 1. Hypothesized biosynthetic pathway and determination of [$^2H_7$]PPA production in rose protoplasts. (A) AADC route (grey arrow) and proposed AAAT route (solid arrow) for the production of 2PE from L-Phe in the isolated protoplasts of *R.* 'Yves Piaget'. The novel AAAT-route involves the hypothetical intermediate PPA and the enzyme RyAAAT. (B) Typical rose petal protoplasts (scale bar in 20 μm). (C) LC–MS chromatogram of [$^2H_7$]PPA in protoplasts. [$^2H_7$]PPA (*m/z* 170 [M−H]$^-$, with asterisk) was detected at a retention time (6.66 min), which was identical to that of authentic PPA (asterisk, *m/z* 163 [M−H]$^-$). [$^2H_7$]PPA was not detected in the protoplasts without (w/o) L-[$^2H_8$]Phe feeding.

it has been reported that aminotransferases play a critical role in forming the corresponding keto-acids that serve as substrates for multiple biochemical reactions (Marilley and Casey, 2004).

Recently it has been reported that PAld and 2PE emission increased when PPA is administered to melon (*Cucumis melo*) cubes (Gonda et al., 2010). *C. melo* aromatic amino acid aminotransferase (AAAT) cDNA was identified from melon EST database and it was confirmed that *C. melo* AAAT converted L-Phe and L-tyrosine to PPA and 4-hydroxyphenylpyruvic acid, respectively. We hypothesize that in rose petals an alternative biosynthetic pathway to produce 2PE from L-Phe *via* PPA exists, the Ehrlich pathway. To confirm the 2PE biosynthetic pathway *via* PPA and identify AAAT in rose petals, we have first used the rose petal protoplasts for feeding experiments with stable isotope-labeled precursors. Tracer experiments in native plants with stable isotope-labeled precursors have long been used to uncover biochemical pathways (Boatright et al., 2004; Hayashi et al., 2004). However, several parameters such as the feeding method, environmental factors, and difference between individual plants may influence the elucidation of biochemical pathways of target compounds and their quantitative analysis. In particular, comparatively high concentrations of labeled precursors like amino acids and organic acids are used to enhance the visualization of target compounds, which may lead to false results. Additionally, we encountered the limitations of detecting the intermediates of metabolic pathways due to the dilution of isotope-labeled compounds with endogenous metabolites (Sayama, 2008). Based on the above considerations, we previously developed a simple and controllable approach to elucidate the biosynthesis of 2PE in rose using isolated rose petal protoplasts and confirmed the incorporation of ^{13}C-labeled shikimic acid into 2PE (Yang et al., 2009). Although isolated protoplasts are an artificial system, this model should reveal some fundamental information regarding the biogenesis of 2PE due to the higher conversion rate of exogenously applied precursors within a short incubation period.

Here we report the data obtained by feeding of L-[$^2H_8$]Phe to protoplasts, which resulted in the conversion to [$^2H_7$]PPA within a short period. Followed by the detection of [$^2H_7$]PPA after the feeding with L-[$^2H_8$]Phe, we have cloned AAATs from rose petals and identified three full length cDNAs of rose AAATs (RyAAAT1-3). Furthermore we characterized biochemically the recombinant RyAAATs, catalyzing the transamination from L-Phe to PPA in 2PE biosynthesis in isolated rose protoplasts.

Materials and methods

Plant material and protoplasts feeding experiments

Cut flowers of Damask rose *Rosa* 'Yves Piaget', grown in the green house, were purchased from Ichikawa Rosary in Mishima-City, Japan. The stages of floral growth and the preparation of protoplasts have been described previously (Hayashi et al., 2004; Yang et al., 2009). L-Phe and L-[$^2H_8$]Phe (2.5 μmol) were dissolved in protoplast buffer and added to the protoplasts. The protoplasts were incubated at 30 °C for 24 h. [$^2H_7$]PPA was extracted and characterized by LC–MS. For 2PE analysis, ethyldecanoate in methanol (1.55 nmol) was added as an internal standard. The volatiles were extracted twice with 700 μL of hexane–ethyl acetate (1:1, v/v). The organic layer was dried over Na_2SO_4 and subjected to GC–MS analyses.

Chemicals

L-[2,3,3,2′,2,′4′,5′,6′-$^2H_8$]Phe (98 at% ^2H) was purchased from Sigma–Aldrich. All the other chemicals were of the highest grade commercially available from Wako Pure Chemicals (Osaka, Japan) and Sigma–Aldrich (Tokyo, Japan), unless noted otherwise.