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Short Communication

Aromatic amino acid decarboxylase is involved in volatile phenylacetaldehyde production in loquat (*Eriobotrya japonica*) flowers

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Abstract Post anthesis, loquat flowers emit volatile benzenoids, including phenylacetaldehyde, phenylethyl alcohol, and 2-phenethyl benzoate. Previous studies have shown that pyridoxal phosphate-dependent aromatic L-amino acid decarboxylase (AADC) produces phenylacetaldehyde from L-phenylalanine. Here, two *AADC* genes (*EjAADC1* and *EjAADC2*) were isolated from loquat (*Eriobotrya japonica*) flowers. The EjAADC1 and EjAADC2 proteins showed approximately 72% and 55% identity, respectively, to a rose AADC homolog that has phenylacetaldehyde synthase activity. Transcript analyses indicated that *EjAADC1* was specifically expressed in petals, with the highest level of expression in fully opened flowers; the petals showed high levels of volatile benzenoids, including phenylacetaldehyde. In contrast, *EjAADC2* was expressed at a lower level than *EjAADC1* in all tested tissues, including leaves and developing flowers. Functional characterization of a recombinant EjAADC1 protein expressed in *Escherichia coli* showed that it catalyzes the formation of phenylacetaldehyde from L-phenylalanine in a pyridoxal phosphate-dependent manner. Our results suggest that *EjAADC1* is mainly responsible for the biosynthesis of volatile benzenoids in loquat flowers.

Key words: aromatic amino acid decarboxylase, loquat, phenylacetaldehyde, volatile benzenoid.

Plants produce a diverse array of volatile compounds for attracting pollinators or for chemical defense against herbivores. Phenylacetaldehyde, phenylethyl alcohol, and 2-phenethyl benzoate are prominent scent compounds in a range of flowers, including petunias and roses (Boatright et al. 2004; Spiller et al. 2010). These compounds are also important components of the aroma of fruits such as tomato and grape and in tea (Fu et al. 2015; Ma et al. 2001; Pan et al. 2012). In tomato fruit, an aromatic L-amino acid decarboxylase (AADC; SlAADC1A) catalyzes the pyridoxal-5′-phosphate (PLP)-dependent decarboxylation of L-phenylalanine (Phe) to form phenylethylamine, which is further metabolized into phenylacetaldehyde by unknown enzymes (Tieman et al. 2006, 2007). In contrast, in petunia flowers, the bifunctional enzyme phenylacetaldehyde synthase (PhPAAS), which belongs to the AADC family, converts Phe to phenylacetaldehyde via decarboxylation and oxidative deamination (Kaminaga et al. 2006). Similarly, rose flowers produce phenylacetaldehyde from Phe using the phenylacetaldehyde synthase RhAADC, but also have an alternative pathway leading to phenylacetaldehyde via phenylpyruvate formation (Hirata et al. 2016). In Arabidopsis, aromatic aldehyde synthase (AAS; AtAAS), which has high homology to AADCs, catalyzes the decarboxylation of Phe into phenylacetaldehyde (Gutensohn et al. 2011). Recently, AASs have been identified in *Medicago truncatula* and *Cicer arietinum*, and these enzymes have decarboxylation activities against several hydrophobic amino acids (phenylalanine, methionine, tryptophan, and leucine), although it is not yet known whether they produce phenylacetaldehyde *in planta* (Torrens-Spence et al. 2014). Since the first isolation of *PAAS* genes in *Petunia hybrida* cv. Mitchell, several other genes responsible for phenylacetaldehyde biosynthesis have been characterized at the biochemical and molecular levels (Gutensohn et al. 2011).

We previously reported that phenylacetaldehyde, phenylethyl alcohol, and 2-phenethyl benzoate are characteristic scent components of loquat (*Eriobotrya japonica*) flowers (Koeduka et al. 2016a, 2016b; Kuwahara et al. 2014). However, the gene responsible

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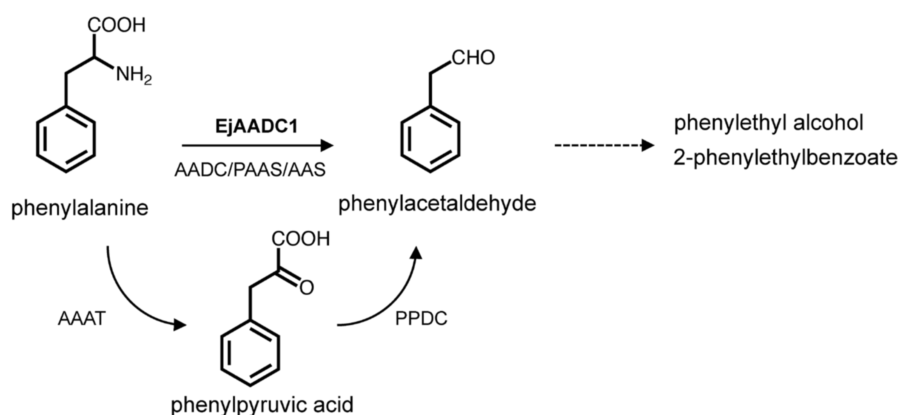

Figure 1. Biosynthesis of volatile benzenoid phenylacetaldehyde in higher plants. Proposed biosynthetic pathway leading to phenylacetaldehyde and its derivatives found in loquat flowers. AADC, Aromatic amino acid decarboxylase; PAAS, Phenylacetaldehyde synthase; AAS, aromatic aldehyde synthase; AAAT, aromatic amino acid aminotransferase; PPDC, phenylpyruvic acid decarboxylase.

for biosynthesis of volatile phenylacetaldehyde in loquat has not been identified to date. In the present study, we performed functional analyses on a loquat cDNA encoding an AADC, that converts Phe to phenylacetaldehyde in a PLP-dependent manner, and contributes to the production of the floral scent of loquat (Figure 1).

To identify putative loquat *AADC* genes, PAAS protein sequences from *P. hybrida* were used as protein queries to search an *E. japonica* database constructed previously (Koeduka et al. 2016b). This homology search identified two AADC-like sequences. These two proteins show 58% identity and were designated EjAADC1 (accession no. LC310891) and EjAADC2 (accession no. LC310892). Phylogenetic analysis with the previously reported AADC protein sequences was performed using MEGA6 software (Tamura et al. 2013) and the neighbor-joining method with 1000 bootstrap replications. Phylogenetic analysis of the characterized AADC proteins indicated that EjAADC1 is closely related to RhAADC (72% identity) and to PhPAAS (66% identity) (Figure 2A). However, EjAADC2 showed high similarity to Arabidopsis AAS and L-tyrosine decarboxylase (TYDC) with 80% and 68% identities, respectively, whereas 55–56% identities were found to RhAADC and PhPAAS. EjAADC1 and EjAADC2 contain a conserved pyridoxal 5′-phosphate-binding lysine residue (Facchini et al. 2000) and a key phenylalanine residue. These two amino acid residues are conserved in all AADC proteins showing acetaldehyde synthase activity except for petunia PhPAAS (Torrens-Spence et al. 2013) (Figure 2B).

RNA-seq analysis, performed as described by Koeduka et al. (2016b), showed that the RPKM (Reads Per Kilobase of transcript per Million mapped reads) values of *EjAADC1* were 75-fold higher than those of *EjAADC2* in flower petals (Figure 3A). The expression of the *EjAADC* genes was analyzed by a semi-quantitative RT-PCR using a 12.5 μl reaction mixture and the manufacturer's PCR program with rTaq DNA polymerase (Toyobo, Japan). Total RNA was isolated from the leaves and from flowers at different development stages (flower bud, open flower just before anthesis, and fully open flower; Koeduka et al. 2016b). As an endogenous standard, loquat *actin* (*EjACT*, accession no. JN004223) was amplified to confirm the use of approximately equal amounts of input cDNA. The gene specific primers used in this experiment were as follows: forward primer 5′-TCT TCA CCA CTC TCG ACT GTT G-3′ and reverse primer 5′-CCA CAA TCT CAA ACC TTT TAT CC-3′ for *EjAADC1*; forward primer 5′-GTC CAG GCG CTG TCT ACA AAT C-3′ and reverse primer 5′-CAG TTT CGT CGC ATA AGT TTC ATC-3′ for *EjAADC2*; forward primer 5′-AAT GGA ACT GGA ATG GTC AAG GC-3′ and reverse primer 5′-TGC CAG ATC TTC TCC ATG TCA TCC CA-3′ for *EjACT*. Semi-quantitative RT-PCR analysis showed that *EjAADC1* was specifically expressed in floral tissues and increased when the flowers bloomed; *EjAADC2* was expressed in all tissues tested (Figure 3B). A detailed expression analysis using quantitative RT-PCR indicated that the transcript levels of *EjAADC1* were approximately 650-fold higher during the post anthesis period (flowering stage-3) than in buds (flowering stage-1) (Figure 3C). Quantitative RT-PCR was performed using the forward primer 5′-ATA TGC CGG GAG TGC TTG CAT T-3′ and the reverse primer 5′-AAC CAT TTG TGC GCG TTC AGA C-3′ in triplicate on a Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) under the following conditions: 30 s at 95°C; followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The amplification was performed using a 15 μl reaction mixture with the KAPA SYBR FAST qPCR Kit (Nippon Genetics Co., Ltd., Tokyo, Japan). To normalize the data, *EjACT* was amplified using the forward primer 5′-AAT GGA ACT GGA ATG GTC AAG GC-3′ and the

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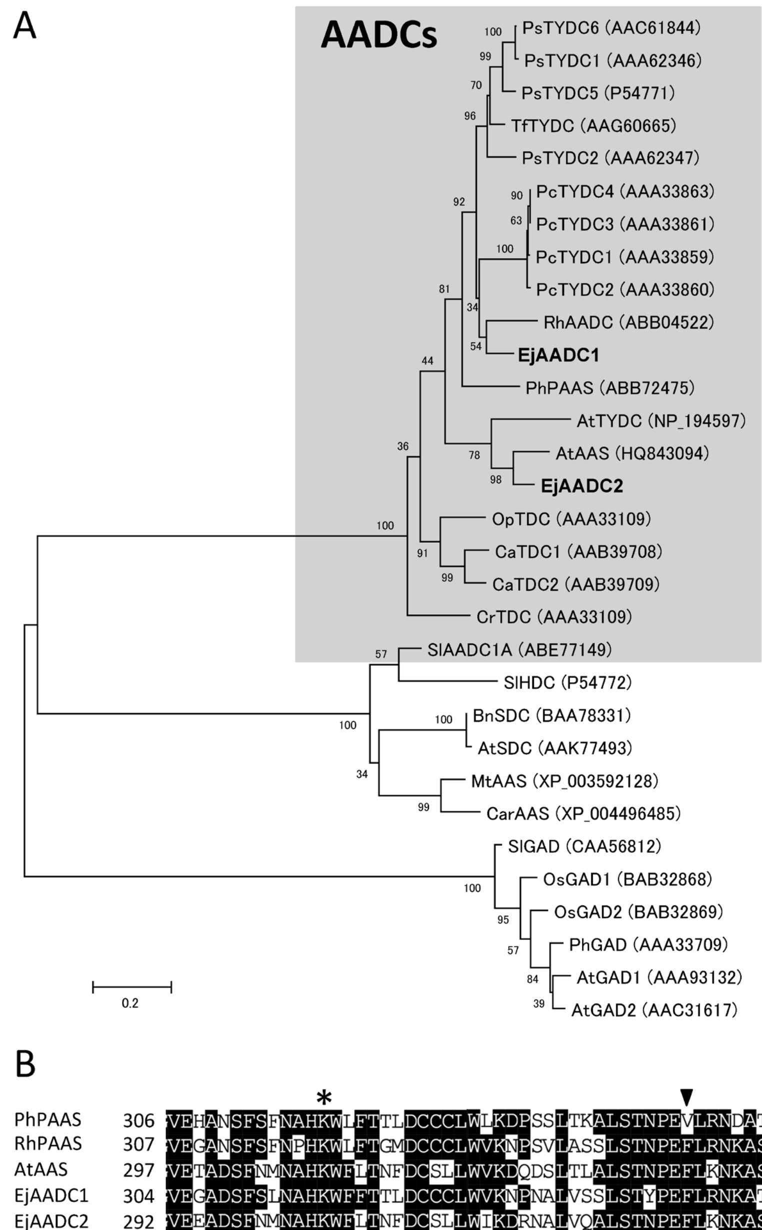

Figure 2. Sequence analysis of EjAADCs. (A) A phylogenetic tree of two loquat AADC genes and other related amino acid decarboxylase genes of different plant species. Phylogenetic analysis was performed in MEGA6 using the neighbor joining method. The scale bar represents 0.2 amino acid substitutions per site. At, Bn, Ca, Cr, Os, Op, Pc, Ph, Ps, Rh, Sl, Tf, Mt, and Car indicate *Arabidopsis thaliana*, *Brassica napus*, *Camptotheca acuminata*, *Catharanthus roseus*, *Oryza sativa*, *Ophiorrhiza pumila*, *Petroselinum crispum*, *Petunia hybrida*, *Papaver somniferum*, *Rosa hybrida*, *Solanum lycopersicum*, *Thalictrum flavum*, *Medicago truncatula*, and *Cicer arietinum*, respectively. AAS, GAD, SDC, TDC, PAAS, TYDC, AADC, and HDC indicate acetaldehyde synthase, glutamate decarboxylase, serine decarboxylase, tryptophan decarboxylase, phenylacetaldehyde synthase, tyrosine decarboxylase, aromatic amino acid decarboxylase, and histidine decarboxylase, respectively. The AADC family is indicated by grey shading. (B) Sequence alignment of EjAADC and known phenylacetaldehyde synthesizing proteins. The conserved pyridoxal 5′-phosphate-binding residue is indicated by an asterisk. The triangle indicates the important residue for aldehyde synthase activity.

reverse primer 5′-TGC CAG ATC TTC TCC ATG TCA TCC CA-3′. The temporal and spatial expression patterns of *EjAADC1* were consistent with the intracellular levels of volatile benzenoids, including phenylacetaldehyde and phenylethyl alcohol during flower development (Koeduka et al. 2016b). Therefore, EjAADC1 was characterized in further enzymatic assays.

To test the in vitro enzymatic activity of EjAADC1, a recombinant protein was heterologously produced in *Escherichia coli*. A DNA fragment encoding the full-length *EjAADC1* gene was amplified by PCR with the primers 5′-GGATCCGGA TGG GTA GCC TCA ATT CAC AA-3′ (the underline indicates a *BamH*I site) and 5′-CTCGAGTCA ATA CTT GAT AAG GAT TTG AT-3′ (the underline indicates an *Xho*I site), and ligated into the pGEM-T easy vector (Promega Co.,

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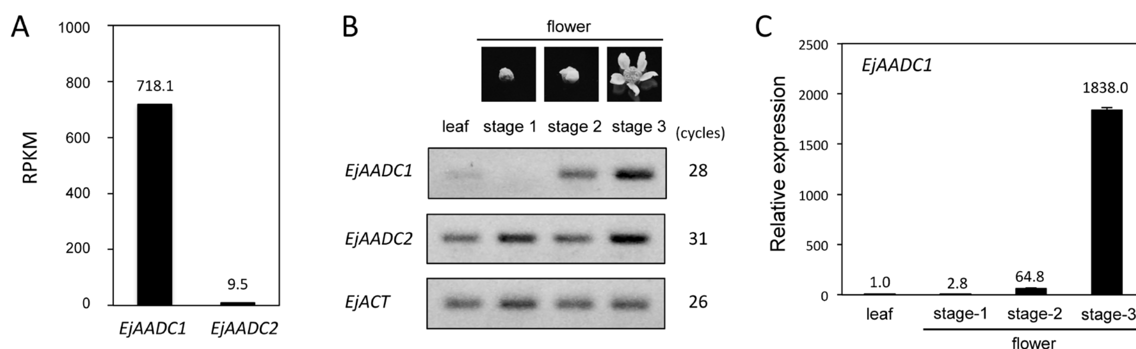

Figure 3. Expression of *EjAADC* genes. (A) RPKM values of *AADC* genes from loquat flowers by RNA-Seq. (B) Tissue specific expression of *EjAADC* genes examined with semi-quantitative RT-PCR. (C) Tissue specific expression of *EjAADC1* gene examined by quantitative RT-PCR.

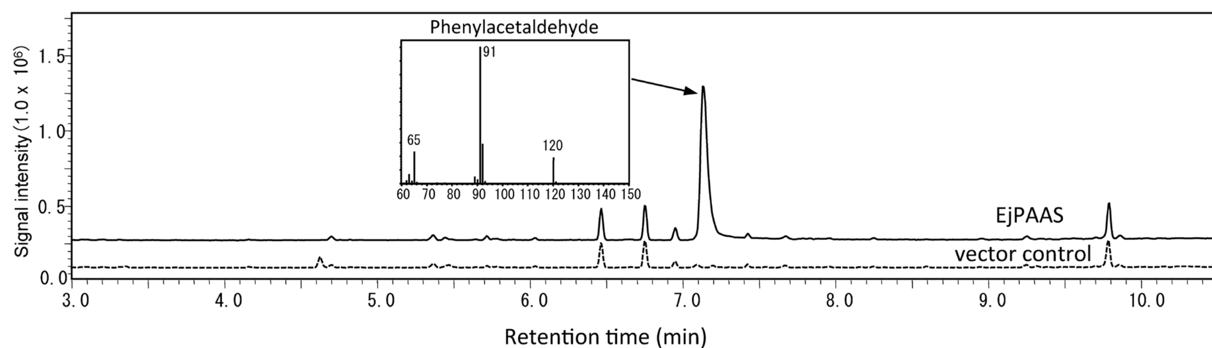

Figure 4. Conversion of phenylalanine to phenylacetaldehyde by EjAADC1. Product analysis of the reaction catalyzed by EjAADC1 by GC-MS.

Madison, WI, USA). After sequencing the amplified fragment, *EjAADC1* was subcloned into the pENTR1A vector and inserted into the expression vector pHis9 using the Gateway cloning systems to produce the target protein fused to an N-terminal nona-histidine tag (Varbanova et al. 2007). The resulting plasmid pHis9-EjAADC1 was transformed into the expression host *E. coli* Rosetta2(DE3)pLysS cells (Merck Millipore). The *E. coli* cells were cultured in 2× YT medium containing 50 mg/L kanamycin and 30 mg/L chloramphenicol at 37°C to an optical density (600 nm) of 0.6; they were then induced with 0.5 mM isopropyl-β-D-thiogalactoside, and allowed to grow for an additional 20 h at 14°C. Bacterial cells were harvested by centrifugation, resuspended in lysis buffer (100 mM HEPES, pH 7.0; 2 mM PLP; 14.3 mM β-mercaptoethanol), and lysed by sonication. The EjAADC1 protein was purified from the *E. coli* lysate by affinity chromatography using the MagneHis Protein Purification System (Promega). Purified recombinant EjAADC1 was analyzed by SDS-PAGE and estimated as 58 kDa. This was consistent with the calculated size of recombinant EjAADC1.

To determine whether EjAADC1 has PAAS activity, an enzyme assay was performed in a 0.4 ml reaction containing 50 mM Tris-HCl (pH 7.0), purified EjAADC1 (17.6 μg of protein), 5 mM PLP, and 5 mM Phe at 30°C for 7 h. The reaction product was extracted with hexane (1.0 ml), concentrated under N_2 gas and analyzed using gas chromatography-mass spectrometer (GC-MS) (QP-5050, Shimadzu, Kyoto, Japan) equipped with a DB-WAX column (30 m length ×0.25 mm diameter ×0.25 μm film thickness, Agilent Technologies). The column temperature was programmed as follows: 60°C for 2 min, increasing by 10°C min^{-1} to 170°C, and subsequently increasing by 20°C min^{-1} to 240°C for 10 min with He as the carrier gas. The mass spectrometer was operated in the electron ionization mode with ionization energy of 70 eV, and ion source and interface temperatures of 240°C with a continuous scan in the range *m*/*z* 60–300. In order to identify reaction products, retention time and mass spectrometer profiles of authentic phenylacetaldehyde were used. GC-MS analysis revealed that EjAADC1 catalyzed the formation of phenylacetaldehyde from Phe, whereas the empty vector control did not produce any phenylacetaldehyde (Figure 4).

It has been reported that loquat flowers produce volatile benzenoids, including phenylacetaldehyde, phenethyl alcohol, and 2-phenethyl benzoate (Koeduka et al. 2016b), whereas loquat leaf volatiles are mostly composed of C6 compounds such as (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenyl butanoate (Kuwahara et al. 2014). The levels of the *EjAADC1* transcript were higher in loquat flowers than in leaves and increased throughout flower development. This spatial and temporal transcript accumulation pattern is consistent with the observed

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changes in phenylacetaldehyde levels. Therefore, it is a reasonable assumption that EjAADC1 predominantly contributes to the formation of phenylacetaldehyde that may function as a pollinator attractant in loquat flowers (Huber et al. 2005). However, we should note that the present data are insufficient to determine whether EjAADC1 was solely responsible for the biosynthesis of phenylacetaldehyde in loquat flowers. In contrast to *EjAADC1*, *EjAADC2* transcripts were detected in all tissues analyzed and thus EjAADC2 may also have a role in phenylacetaldehyde formation in developing flowers.

The previously identified rose RhAADC and petunia PhPAAS were shown to be members of the pyridoxal phosphate-dependent AADC family of decarboxylases, which also includes AtAAS and SlAADC1A. Our phylogenetic analyses indicate that EjAADC1 falls into the same clade as RhAADC, whereas EjAADC2 is closely related to an Arabidopsis clade that contains proteins biochemically characterized as possessing AAS and tyrosine decarboxylase activities (Gutensohn et al. 2011; Lehmann and Pollmann 2009) (Figure 2A). Although Gutensohn et al. (2011) reported that AtAAS contributes phenylacetaldehyde formation *in planta*, AtAAS shows the higher enzymatic activity toward L-Dopa rather than Phe. This suggests that the catalytic property of AtAAS is different from that of PhPAAS and RhAADC. Thus, the ancestral proteins of EjAADC1 and RhAADC with PAAS activity evolved independent of AtAAS and EjAADC2, although we have not yet tested the PAAS activity of EjAADC2.

Hirata et al. (2016) described the two different biosynthetic pathways for the production of phenylacetaldehyde from Phe in rose flowers. In addition to the biosynthetic pathway involving RhAADC, rose flowers produced phenylacetaldehyde by an alternative biosynthetic pathway involving two sequential reactions catalyzed by an aromatic amino acid aminotransferase (AAAT) and phenylpyruvic acid decarboxylase (PPDC) (Figure 1). A search of the loquat database identified one loquat cDNA that encodes proteins with 89% identity to rose AAAT, and three loquat cDNAs encoding proteins with 83–92% identities to rose PPDC. This suggests that loquat flowers may possess an alternative biosynthetic pathway for phenylacetaldehyde in a similar manner as rose flowers. It thus appears that two pathways have evolved in the Rosaceae family.

Overall, our study has provides biochemical evidence showing that loquat produces the floral scent compound phenylacetaldehyde via EjAADC1 with PAAS activity. Thus, EjAADC1 is a new member of the AADC family for biosynthesis of floral scent compounds and may be useful as a molecular tool for in vivo phenylacetaldehyde production in heterologous expression strategies in future genetic engineering studies.

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