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

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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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56 ABSTRACT

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

route and the new route *via* PPA from L-Phe.

Keywords

2-Phenylethanol; Aromatic amino acid aminotransferase; Biosynthesis; Phenylpyruvic acid; Rose

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

Introduction

2-Phenylethanol (2PE) is one of the prominent scent compounds produced by Damask roses (Hayashi et al., 2003; 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,

113 AADC and PAR in plants.

114 Microorganisms **biosynthesize** 2PE from L-Phe *via* phenylpyruvic acid (PPA),
115 called ‘Ehrlich pathway’ (Ehrlich, 1907), while there is no **report** about the Ehrlich
116 pathway in *planta* so far. In microorganisms, the amino **acid** metabolism has been
117 studied in detail, **and it has been reported that** aminotransferases play a critical role in
118 forming the corresponding keto-acids that serve as substrates for multiple biochemical
119 reactions (Marilley et al., 2004).

120 Recently it has been reported **that PAld and 2PE emission increased** when **PPA**
121 **is administered to melon (*Cucumis melo*) cubes (Gonda et al., 2010).** *C. melo*
122 aromatic amino acid aminotransferase (AAAT) cDNA was identified from melon EST
123 database and it was confirmed that *C. melo* AAAT converted L-Phe and L-tyrosine to
124 PPA and 4-hydroxyphenylpyruvic acid, respectively. We hypothesize that **in** rose
125 petals **an alternative biosynthetic pathway to produce 2PE from L-Phe *via* PPA exists,**
126 **the** Ehrlich pathway. To confirm the 2PE biosynthetic pathway *via* PPA and identify
127 AAAT in rose **petals,** we have first **used the rose petal protoplasts for feeding**
128 **experiments with stable isotope-labeled precursors. Tracer experiments in native**
129 **plants with stable isotope-labeled precursors have long been used to uncover**
130 **biochemical pathways** (Boatright et al., 2004; Hayashi et al., 2004). However, several
131 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- $[\text{}^2\text{H}_8]\text{Phe}$ to protoplasts, which resulted in the conversion to $[\text{}^2\text{H}_7]\text{PPA}$ within a short period. Followed by the detection of $[\text{}^2\text{H}_7]\text{PPA}$ after the feeding with L- $[\text{}^2\text{H}_8]\text{Phe}$, 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.

Material 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-[²H₈]Phe (2.5 μmol) were dissolved in protoplast buffer and added to the protoplasts. The protoplasts were incubated at 30 °C for 24 h. [²H₇]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 Na₂SO₄ and subjected to GC-MS analyses.

Chemicals

L-[2,3,3,2',2',4',5',6'-²H₈]Phe (98 atom% 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.

170 Determination of [$^2\text{H}_7$]PPA by LC-MS

171 The protoplasts were administered with L- $^2\text{H}_8$ Phe (2.5 μmol , 24 h at 30 $^\circ\text{C}$)
172 and then lyophilized. The lyophilized powder was dissolved in 15 mL Milli-Q water
173 and applied to an SPE cartridge (SupelcleanTM ENVI-Chrom P SPE Tube, 500 mg).
174 The cartridge was washed with 6 mL of water and eluted with 6 mL of acetonitrile.
175 The acetonitrile fraction was concentrated *in vacuo* and redissolved in 100 μL 5%
176 acetonitrile. An aliquot of each sample was subjected to the LC-MS analysis. An
177 authentic sample of PPA showed retention time of 6.66 min and m/z 163 $[\text{M-H}]^-$ while
178 $^2\text{H}_7$ PPA showed a retention time of 6.62 min and m/z 170 $[\text{M-H}]^-$.

179

180 Preparation of crude enzymes from the flowers of *R. 'Yves Piaget'*

181 Preparation of crude enzymes has been described previously (Sakai et al.,
182 2007). The detailed conditions are described in the 'Supplementary information'.

183

184 Assay of L-Phe transamination activity

185 Reaction mixture (150 μL) containing 10 mM L-Phe, 10 mM α -ketoglutaric
186 acid and 30 μL enzyme solution in a 0.5 M Tris-HCl buffer (pH 9.0) was incubated at
187 45 $^\circ\text{C}$ for 10 min. The reaction was stopped by adding an equal volume of acetonitrile
188 after adding 200 nmol L- $^2\text{H}_8$ Phe as an internal standard. The sample was subjected

to centrifugation (20,000 g, 10 min, 4 °C), and filtered (Millex LH, Millipore) prior to analysis by LC-MS. For the reverse reaction 10 mM PPA and 10 mM L-glutamic acid were used instead of L-Phe and α -ketoglutaric acid identical reaction conditions.

Assay of PPA decarboxylation activity

Reaction mixture (200 μ L) containing 5 mM PPA, 0.1 mM Thiamine diphosphate, 0.5 mM $MgCl_2$, 100 μ l crude enzyme solution in a 0.2 M citrate buffer (pH 6.0), and was incubated at 35 °C for 2 h. Then 3-phenylpropion aldehyde (23.3 pmol) dissolved in ethanol (as an internal standard) was added to reaction mixture and PPA was extracted with a 400 μ l mixture of hexane and ethylacetate (1:1 v/v). The organic phase was dried over anhydrous Na_2SO_4 and analyzed by GC-MS.

LC-MS analysis

The LC-MS separation module was equipped with a SHIMADZU SPD-M10A PDA and LC-MS2010A. The separation was performed on a YMC-Pack ODS AQ column (2.0 $\times$ 150 mm, 5.0 μ m, YMC) connected to a GUARD CARTRIDGE CAPCELL C18 UG120 (4.0 $\times$ 10 mm, SHISEIDO). The solvents used were A: 5 mM ammonium acetate (pH 7.0), and B: acetonitrile (Dorman et al., 2008). The gradient was developed by increasing the latter from 5% to 90% in 13.5 min, then holding the

concentration constant for 2.5 min with a flow rate of 0.2 mL/min at 40 °C. The PDA detection range was 190 to 370 nm and the MS detection system was operated in the negative ionization mode with a scan range of m/z 100–300. The tuning parameters were as follows: capillary voltage 3.5 kV; cone voltage 20 V; source block temperature 120 °C; and desolvation temperature 350 °C.

GC-MS analysis

The volatile compounds were analyzed as described previously (Yang et al., 2009). The details conditions are described in the ‘Supplementary information’.

EST database of rose flowers

Rose flower petals (stage 5, 5 g) were subjected to RNA extraction with RNeasy Plant Mini Kit (QIAGEN) and then purified to obtain mRNA with Oligotex-dT30 mRNA Purification Kit (TaKaRa). EST database was constructed by Dragon Genomics Center (TaKaRa Bio, Japan).

Molecular cloning of RyAAATs and expression in *E. coli*.

Degenerate RyAAATs primers (Supplementary Table 1) were designed using conserved regions among aspartate aminotransferases (AspATs, GenBank association

227 numbers: NP001031394, NP850022, NP565529, AAQ54557, BAD54126,
228 BAD27593, BAD19094, NP178152, NP177890, NP001118421, and NP180654), and
229 the tyrosine aminotransferase (TyrAT) EST (CF349437 in *R. hybrid* cultivar)
230 (Guterman et al., 2002) and the alanine aminotransferase (AlaAT) (in-house rose EST
231 database). These cDNAs of RyAAAT candidates were amplified by RT-PCR from
232 total RNA extracted from stage 3 rose petals. The PCR products were cloned into the
233 pCR2.1 TA-Cloning vector (Invitrogen) and sequenced, and then 3 different
234 sequences of RyAAAT candidates were subcloned into the pET28a expression vector,
235 which contains an N-terminal histidine tag (Novagen). The cloned genes were
236 expressed in *E. coli* BL21(DE3) cells grown in LB medium with 50 µg/mL
237 kanamycin at 37 °C. Protein production was induced by the addition of 1 mM IPTG
238 (TaKaRa). After incubation at 27 °C for 24 h, the crude proteins were extracted with
239 20 mM potassium phosphate buffer (pH 7.5) containing 0.75 mM PLP and 0.1%
240 Triton X-100 (Extraction buffer). The proteins were purified with HisTrap HP column
241 (GE Healthcare) equilibrated with the extraction buffer (without Triton X-100) and
242 eluted using a linear 0–0.75 M imidazole gradient in the same buffer. Protein
243 concentrations were determined using Bradford reagents (Bio-Rad) with bovine
244 serum albumin as the standard.

245

246 RyAAAT3 substrate specificity

247 The substrate specificity for transamination of RyAAAT3 was determined by
248 quantification of the various keto-acid products and the reduction of L-alanine and
249 glycine. [The details are described in the 'Supplementary Information'](#).

250

251 Inhibition of PLP-dependent enzymes with Carbidopa

252 We have reported rose AADC expression in *E. coli* (Sakai et al., 2007). The
253 crude proteins were extracted with 20 mM potassium phosphate buffer (pH 7.5)
254 containing 1 mM PLP and 0.1% CHAPS. The proteins were purified as described in
255 RyAAATs expression. AADC assay mixtures (400 μ L) containing 4 mM L-Phe and
256 200 μ L enzyme solution in a 0.2 M potassium phosphate buffer (pH 8.0) were
257 incubated at 35 °C for 1 h, and then PAld was extracted and analyzed [as](#) described
258 above.

259 Inhibition assays of AADC and AAAT with Carbidopa (Burkhard et al., 2001;
260 Bertoldi et al., 2002) were performed as decarboxylation and transamination assay
261 with or without carbidopa dissolved in [dimethylsulfoxide \(DMSO\) to make a final](#)
262 [concentration of 5%.](#)

263

264 Transcription analysis of *RyAAAT3*

Total RNA was extracted from various tissues derived from three different plants of *R. 'Yves Piaget'* at stage 4. RT-PCR was performed using primer sets for *RyAAAT3* and 18S *rRNA* (Ambion) as a house keeping gene. Competitor primers for 18S *rRNA* were used to avoid excess amplification of the 18S *rRNA*. The ratio of 18S *rRNA* primers and competitor primers was 4:6 (v/v). The transcripts were visualized on a 1% agarose gel stained with ethidium bromide and the amplification curves were at linear process as confirmed at various cycles. *RyAAAT3* primers are listed in Supplementary Table 1.

RNAi suppression experiments

Two kinds of double strand RNA (dsRNA) were prepared based on *RyAAAT3* partial sequences. The dsRNA of *RyAAAT3* were synthesized at positions 56-701 of 1266 bp using the T7 RiboMAX System (Promega) and specific primers (Supplementary Table 1), and annealed by incubation at 70 °C for 10 min (An et al., 2003; 2005). The rose protoplasts (3×10^5 cells in 200 μ L protoplast buffer) were transfected with 75 μ g dsRNA using an equal volume of 40% polyethylene glycol-calcium solution (PEG 6000, Fluka) (Sheen, 2001). After incubation for 24 h at 30 °C, 2.5 μ mol L-Phe was added to the protoplasts and they were incubated for another 24 h at 30 °C. PPA was not detected in the protoplasts, and PAld was detected in low level.

The variation of PAld was too large due to its quick conversion to 2PE in the protoplasts, and therefore the effects of RNAi were evaluated based on the amounts of 2PE. 2PE extraction and GC-MS analysis were carried out as described in the section, 'GC-MS analysis'. Effect of RyAAAT3 knockdown was confirmed by RT-PCR as described above.

Results

L-Phe was converted to PPA, and PAld was produced from PPA

To confirm the transamination from L-Phe to PPA in rose petal protoplasts (Fig. 1B), we analyzed [$^2\text{H}_7$]PPA by LC-MS after administration of L-[$^2\text{H}_8$]Phe to rose petal protoplasts. Authentic PPA (m/z 163 [M-H] $^-$) was detected at a retention time (6.66 min), together with an ion peak detected at 6.62 min of m/z 170 [M-H] $^-$ for [$^2\text{H}_7$]PPA (Fig. 1C). These results demonstrated that PPA was biosynthesized in protoplasts from L-Phe.

We also determined the transamination activity from L-Phe to PPA and conversion from PPA to PAld in the crude enzymes prepared from rose petals. PPA was detected by LC-MS based on the detection of peaks of the ion trace at m/z 163 [M-H] $^-$ with the same retention time of authentic PPA (Fig. 2A), revealing that L-Phe was converted to PPA by the enzymes in the *R. 'Yves Piaget'*. Furthermore, we

detected PAld production in the crude enzymatic assay mixture with PPA as a substrate by GC-MS (Fig. 2B), revealing that PPA was converted to PAld, precursor of 2PE.

Full lengths of three RyAAATs were determined and RyAAAT3 showed AAAT activity with L-Phe

Blast searching and degenerate PCR cloning from rose petals gave us three EST candidates of RyAAAT putatively catalyzing transamination of L-Phe. We separately obtained three independent full length cDNAs by 3', 5'-RACE PCR. To elucidate whether these cDNAs (RyAAAT1, RyAAAT2 and RyAAAT3) encode functional enzymes the cDNAs were transferred a position vector for expression in *E. coli*. We purified the expressed enzymes and evaluated the activity based upon PPA production. One of candidates (RyAAAT3), homologous to TyrAT, showed PPA production activity with L-Phe (Fig. 3A,B, Supplementary Fig 1). However the other two RyAAAT1 and 2 homologous to AspAT and AlaAT hardly showed any PPA production (Supplementary Fig. 2). Therefore we focused on RyAAAT3 which is a 421 amino acid protein with a calculated average molecular weight of 46.3 kDa and pI of 6.28 (GenBank/EMBL accession number AB669189), and includes a aminotransferases family-I PLP attachment site (SLSKrwLVpGWRLG) and the

322 highly conserved residue (arginine 393) in family I for the binding of these enzymes
323 substrates (Huang et al., 2008; Yennawar et al., 2001). The deduced amino acid
324 sequences of RyAAAT3 showed 78% similarity to *C. melo* AAAT. *In silico* analysis
325 by TargetP 1.1 (<http://www.cbs.dtu.dk/services/TargetP/>), ChroloP 1.1
326 (<http://www.cbs.dtu.dk/services/ChloroP/>), SignalP 4.0
327 (<http://www.cbs.dtu.dk/services/SignalP/>) and WoLF PSORT (<http://wolfpsort.org/>),
328 showed that RyAAAT3 does not contain signal peptides at its *N*-terminal, suggesting
329 cytosolic localization.

330

331 RyAAAT3 preferred α -ketoglutaric acid as the amino-acceptor in the production of
332 PPA than oxaloacetic acid

333 Optimum pH of enzyme was screened in a range of 7-10 by monitoring the
334 production of PPA (Supplementary Fig. 3A). Also, the optimum temperature for the
335 PPA production was determined and the highest at 45 °C was observed
336 (Supplementary Fig. 3B). For RyAAAT3, α -ketoglutaric acid was the preferred amino
337 acceptor rather than oxaloacetic acid in production of PPA from L-Phe under the
338 optimum conditions in pH and reaction temperature conditions (Fig. 4A).
339 Recombinant RyAAAT3 revealed the K_m and V_{max} values for the conversion of L-Phe
340 with oxaloacetic acid were 0.73 ± 0.11 mM, 6.86 ± 0.04 nmol/mg protein/min,

respectively, and with α -keto-glutalic acid were 1.47 ± 0.37 mM, 21.85 ± 0.59 nmol/mg protein/min respectively (means $\pm$ SD; n=4).

RyAAAT3 preferred the forward reaction from L-Phe as a substrate to PPA than the reverse reaction

To further characterize the enzymatic properties of RyAAAT3, we analyzed relative activities of transamination reactions with several amino acids such as L-Phe, D-phenylalanine, L-tyrosine and L-tryptophan. L-Phe gave the highest transaminase activity, on the other hand L-tyrosine and L-tryptophan aromatic amino acids gave one-fourth activity in comparison to L-Phe. RyAAAT3 hardly showed any activity for substrates L-alanine, D-phenylalanine and glycine (Fig. 4B).

Aminotransferases catalyze *bi*-directional reactions in transamination. It was proposed that L-Phe would be produced from PPA by aminotransferase in plants (Maeda et al., 2011). We also determined the reaction selectivity of RyAAAT3 in transamination. Forward reaction (L-Phe as a substrate, α -keto-glutalic acid as an amino acceptor) and the reverse reaction (PPA and L-glutamic acid were used as an acceptor and amino donor, respectively, under the same conditions as the forward reaction) were evaluated by LC-MS analyses for the products (Supplementary Fig. 4). The RyAAAT3 showed 9.7 fold higher transamination activity from L-Phe to PPA

(19.37 $\pm$ 0.67 pmol/mg protein/min) than reverse reaction (2.01 $\pm$ 0.47 pmol/mg protein/min). These results suggested that RyAAAT3 preferred L-Phe as a substrate compared to PPA.

Carbidopa inhibited transaminase activity of RyAAAT3

It is well known that aminotransferases form Schiff bases between the amino group of amino acid residue of aminotransferase and PLP. Also, rose AADC was reported to produce PAld with forming Schiff base. To confirm whether RyAAAT3 is PLP dependent enzyme or not, we quantified the production of PAld and PPA in AADC (decarboxylation) and AAAT (transamination) enzymatic assays with Carbidopa. Carbidopa inhibited production of PAld and PPA in concentration dependent manner and at 500 μ M concentration PAld and PPA were hardly produced in the heterogeneously expressed rose AADC and RyAAAT3 (Fig. 5A,B).

RyAAAT was expressed in rose petals and various organs

We examined transcription level of *RyAAAT3* in various rose tissues at stage 4 by RT-PCR. *RyAAAT3* was expressed in rose petals and also other tissues, leaf, stem, rose hip and calyx (Fig. 6). The *C. melo* AAAT was expressed in similar manner in various organs, shoots, young and old leaves, and in immature fruits (Gonda et al.,

2010). Also, PAAS in petunia and AADC in tomato were expressed in various organs and not only in petals. The RyAAAT3 expressed in the petals may produce PPA from L-Phe in rose petals.

RNA interference of *RyAAAT3* suppressed 2PE production in the rose protoplasts

To elucidate the contributions of *RyAAAT3* to 2PE synthesis in the rose flowers, we performed RNAi experiments targeting *RyAAAT3* in the rose protoplasts. 2PE production in non-treated samples was almost the same as compared to control samples. RNAi experiments towards *RyAAAT3* decreased the 2PE production about 60% as compared to the control samples (Fig. 7). These results suggested *RyAAAT3* plays crucial role in 2PE biosynthesis of in rose flowers.

Discussion

Plant specialized (secondary) metabolites are biosynthesized in plants for survival in diverse ecological niches. These multitudes of chemicals are recognized as defense compounds against biotic and abiotic stresses and also as allelochemicals of other living organisms. Aromatic amino acids, L-tyrosine and L-Phe are initial compounds of the phenylpropanoid biosynthetic pathway leading to phenylpropanoids, lignans, lignins, stilbenes, coumarins, alkaloids and flavonoids. 2PE, a major

398 constituent of scent in many flowers and **fruits is** also derived from L-Phe. The
399 biological functions of 2PE have been considered as an antimicrobial **agent** and also
400 **as** a potent insect attractant (Pichersky and Gershenzon, 2002). Our previous
401 deuterium label feeding experiments in rose (*R. 'Hoh-Jun'* and *R. damascena* Mill.)
402 demonstrated four potential routes producing 2PE from L-Phe *via* i) PAld, ii)
403 phenethylamine, iii) *trans*-cinnamic acid/phenyllactate and iv) PPA (Erich **pathway** in
404 microorganism) (Hayashi et al., 2004). Tieman et al (2006) clearly showed that L-Phe
405 is decarboxylated to phenethylamine by a tomato AADC. Phenethylamine **thus**
406 **produced** is converted to PAld by removal of the amine group, followed by
407 conversion to 2PE by PAR, while one *bi*-functional enzyme decarboxylates and
408 deaminates L-Phe directly producing PAld in petunia and rose flower (Kaminaga et al.,
409 2006; Sakai et al., 2007). **AADCs also play key roles in PAld production from L-Phe**
410 **in herbivory response and floral scent production in *Arabidopsis* plants (Gutensohn et**
411 **al., 2011).**

412 In this report, we demonstrated **an** alternative route to convert L-Phe to PPA and
413 then PPA to PAld with crude enzymes from rose petals. These results indicated L-Phe
414 was converted to PAld *via* PPA **in an enzymatic** reaction in rose flowers. Also, we
415 were successful in detecting [²H₇]PPA in rose petal protoplasts by feeding L-[²H₈]Phe,
416 suggesting **that** the transamination of L-Phe to PPA **is** working *in vivo*. **So far** we could

417 not detect endogenous PPA in rose petal protoplasts by LC-MS. Similarly the
418 endogenous PPA was not determined in petunia though it was measurable after
419 feeding shikimic acid (Maeda et al., 2010). These results suggest that PPA is pretty
420 quickly converted to PAld in plants. Our conversion study here strongly suggested
421 that 2PE is biosynthesized from L-Phe via PPA in rose protoplasts and rose AAAT
422 forms PPA enzymatically from L-Phe. Although we have not identified genes
423 encoding the second step, PPA is presumably converted by a decarboxylase to PAld.
424 As isolated protoplasts are not physiologically and biochemically the same as the
425 flower petals, further investigations with rose petals are needed to confirm the actual
426 roles of AAAT and PPA in the 2PE biosynthesis. Although RyAAAT3 was expressed in
427 the whole rose flower plant, 2PE concentrations were much lower in leaves, stems,
428 and calyxes than in flower petals (Oka et al., 1999). We need to investigate the role of
429 RyAAAT3 in flowers, leaves, stems, and calyxes of the rose plants.

430 AAATs are classified in various AAAT families, such as phenylalanine
431 aminotransferases (PheATs) similarly to tyrosine aminotransferases (TyrATs) and
432 tryptophan aminotransferases (TrpATs) and several reports concerning enzymatic
433 characterizations of AAATs have been published (Lopukhina et al., 2001; Stepanova
434 et al. 2008; Tao et al., 2008; Prabhu and Hudson, 2010; Gonda et al., 2010; Lee and
435 Facchini, 2011). *Arabidopsis* WEAK ETHYLENE INSENSITIVE8 (*WEI8*) gene family

link the tissue-specific effects of ethylene and auxin production (Stepanova et al., 2008) and the shade avoidance response of plants to low red/far-red light is essential for the expression of *SHADE AVOIDANCE3* (*SAV3*) gene (Tao et al., 2008). These two genes, *WEI8* and *SAV3*, encode a TrpAT that catalyzes the conversion of tryptophan to indole-3-pyruvic acid, an intermediate in auxin biosynthetic pathway. Functional characterization of the *Arabidopsis* the locus tags At4g23600 and At5g36160, annotated as TyrAT, revealed that the enzymes form 4-hydroxyphenylpyruvate from L-Tyr. There are a few reports that plant AAATs convert L-Phe to PPA, but the transamination activities for L-Phe of reported plant AAATs are quite low compared to the reaction preferred substrate (Lee and Facchini, 2011). The existence of specific PheAT has remained unclear despite substantial efforts. Very recently, a novel PheAT gene in melon was isolated and characterized (*C. melo* AAAT) encoding 45.6 kDa protein. *C. melo* AAAT catalyzed the transamination of L-Phe and L-Tyr to PPA and 4-hydroxyphenylpyruvic acid, respectively. With low turnovers *Arabidopsis* TyrAT (At5g36160) also formed PPA from L-Phe (Prabhu and Hudson, 2010). Our cloned RyAAAT3 showed high similarity with *C. melo* AAAT at 78% and with *Arabidopsis* TyrAT (At5g36160) at 74%, whereas RyAAAT3 showed relatively low similarity with *Arabidopsis* *WEI8/SAV3* (TrpAT) and poppy TyrAT (Lee and Facchini, 2011) at 50-60%. A phylogenetic analysis showed that RyAAATs and

455 some aminotransferases were classified into three clades, AspAT, AlaAT and
456 aminotransferases specific to aromatic amino acids, based on their amino acid
457 sequences (Supplementary Fig. 5). Similar to RyAAAT3, *C. melo* AAAT expressed in
458 *E. coli* preferred L-Phe as a substrate by 3.5 fold higher than L-tyrosine. We here
459 propose that the RyAAAT3 and the *C. melo* AAAT are ‘phenylalanine
460 aminotransferases’ within the AAAT clade as these aminotransferases were only
461 reported to have the preference for L-Phe as a substrate. A Blast search in the plant
462 EST database (TIGR Plant Transcript Assembly BLAST Server,
463 <http://plantta.jcvi.org/index.shtml>) identified EST clones with homology to RyAAAT3
464 in various plants such as *Arabidopsis thaliana*, *Medicago truncatula*, *Solanum*
465 *lycopersicum* (tomato), *Helianthus annuus* (sunflower), *Fragaria vesca* (woodland
466 strawberry), *Malus x domestica* (cultivated apple), *Glycine max* (soybean), *Vitis*
467 *vinifera* (wine grape), *Coffea canephora* (coffee robusta), *Gossypium raimondii*,
468 *Solanum tuberosum* (potato) and *Brassica napus* (oilseed rape). Although these ESTs
469 are annotated as a TyrAT because a PheAT is very newly reported, this Blast search
470 indicates the possibility that PheAT are widely distributed throughout the plant
471 kingdom.

472 Enzymatic findings demonstrated that 2PE is biosynthesized *via* different
473 pathway compared to previous reports on tomato, petunia and rose. This study is the

first report **establishing** that rose **protoplasts** utilize two different 2PE biosynthetic pathways and that L-Phe is converted to PAld *via* PPA to produce 2PE. It remains unclear why rose flowers utilize two different 2PE biosynthetic pathways. **We need to quantify the contribution of RyAAAT3 in 2PE production in the flowers of *R.* ‘Yves Piaget’.**

Rose AADC may be involved in reproduction process in rose. It reported that the potential biological function of Arabidopsis TyrAT is the defense response to herbivores and pathogens (Song et al., 2004a,b). The mRNAs of Arabidopsis TyrAT could be induced by various octadecanoids and by wounding of the plants and accumulation of the Arabidopsis TyrAT protein was observed after application of chemical and physical stresses (Lopukhina et al., 2001). Moreover, Song et al (2004) demonstrated that two aminotransferases, AGD2 (At4g33680) and ALD1 (At2g13810) is integral genes in the biosynthesis of phytopathogen resistance chemicals. These findings proposed **that** RyAAAT3 may **play** an important role in resistance against various environment stresses in rose, **although further investigations are needed.**

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Legends of figures

Fig. 1. Hypothesized biosynthetic pathway and determination of [$^2\text{H}_7$]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 [$^2\text{H}_7$]PPA in protoplasts. [$^2\text{H}_7$]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] $^-$). [$^2\text{H}_7$]PPA was not detected in the protoplasts without (w/o) L-[$^2\text{H}_8$]Phe feeding.

626

627 Fig. 2. Conversion of L-Phe to PPA and PPA to PAld with a crude enzyme extract. (A)
628 LC-MS chromatograms of L-Phe conversion to PPA. (B) GC-MS chromatograms of
629 PPA conversion to PAld. PPA (retention time = 6.66 min) was traced at m/z 163 [M-
630 H]⁻ on LC-MS, and PAld (retention time = 4.56 min) was traced at m/z 120 [M]⁺ by
631 GC-MS. The asterisks indicated peaks of PPA (A) and PAld (B), respectively.
632 Negative control stands for the chromatograms of the reaction mixtures at the
633 beginning of the enzymatic reaction. PAld production from PPA by chemical
634 conversion was negligible during the incubation period.

635

636 Fig. 3. Heterogeneous expression and transaminase activity of RyAAAT3. (A) SDS-
637 PAGE of samples used in the RyAAAT3 activity analysis and the image of RyAAAT3
638 visualized by Ag-staining (lane 1-3) and His-tag-specific reagent (His-Detect In-Gel
639 Stain, Nacalai Tesque, Kyoto Japan) (lanes 4 and 5). Lane 1, protein molecular
640 markers; lanes 2 and 4, empty vector; lanes 3 and 5, purified recombinant RyAAAT3
641 protein (arrow). (B) LC-MS chromatogram of enzymatic product of RyAAAT3. PPA
642 (asterisks) was detected at 6.66 min on the LC-MS chromatogram monitored at m/z
643 163 [M-H]⁻. Negative control on Fig. 3B indicates the LC-MS chromatogram
644 monitored at m/z 163 [M-H]⁻ for the reaction mixture at beginning of the reaction in

the presence of L-Phe and RyAAAT3.

Fig. 4. Characterization of RyAAAT3 in amino acceptor preference and substrate specificity. (A) RyAAAT3 kinetics with oxaloacetic acid (open circle) or α -ketoglutaric acid (closed circle). (B) Relative conversion of selected amino acids to their keto-acids, catalyzed by RyAAAT3. (Abbreviations are as follows: L-Phe; L-Phenylalanine, L-Trp, L-Tryptophan; L-Tyr, L-Tyrosine; D-Phe; D-Phenylalanine, Gly; Glycine, L-Ala; L-Alanine). Relative activities were determined under L-Phe $V_{\max}$ conditions. The activity (21.85 ± 0.59 nmol/mg protein/min) with L-Phe was taken at 100%. Amino acids were used at equal concentrations of 10 mM. N.D. means not detected. Error bars represent standard deviation (SD, $n=3$).

Fig. 5. Inhibition of PLP-dependent enzymes, rose AADC and RyAAAT3. PAld (A) and PPA (B) productions were examined in the presence of Carbidopa based on the GC-MS and LC-MS analyses, respectively. The amounts of PAld and PPA in the absence of Carbidopa were taken as 1.0 for control group (CNT). N.D. means not detected. The relative rates were calculated based on the averages of three replicates $\pm$ SD.

664 Fig. 6. Transcriptional analysis of *RyAAAT3* in various organs of *R. 'Yves Piaget'*.
665 Total RNA was extracted from tissues of 3 independent *R. 'Yves Piaget'* plants. RT-
666 PCR of 18S ribosomal RNA was used as a house keeping gene. *RyAAAT3* primers for
667 transcriptional analysis are listed in Supplementary Table 1.

668

669 Fig. 7. Effect of RNAi-mediated silencing of *RyAAAT3* in protoplasts. The 2PE
670 synthesis was measured in protoplasts prepared from rose petals. The control (CNT)
671 and knockdown groups (*RyAAAT3* K/D) were treated with PEG-Ca solution, and the
672 NT groups were not treated. Statistics analysis was performed in student *t*-test. The
673 amount ($15 \text{ nmol}/10^5$ protoplast cells) of 2PE production for CNT was taken as 1.0.
674 The relative rates were calculated based on the averages of 2PE amounts in the CNT
675 group and the data were obtained from three independent replicates $\pm$ SD.

Supplementary Information

Material and methods

Preparation of crude enzymes from the flowers of *R. 'Yves Piaget'*

The rose petals were crushed in liquid nitrogen, and lyophilized to obtain powdered material. For the L-Phe conversion, this material (0.7 g) was homogenized in 0.1 M Tris-HCl buffer pH 8.0 containing 0.1 mM PLP, 1 mM EDTA, 1% glycerol and 0.1% TritonX-100 in the presence of 5.3 g PVPP (Polyclar 10, ISP Japan). After centrifugation (26,000g, 15 min, 4 °C), the proteins in the supernatant were precipitated with ammonium sulfate (0-80%). Then additional centrifugation (26,000g, 30 min, 4 °C) gave the pellets, and it was dissolved in Tris-HCl buffer pH 8.0 containing 0.1 mM PLP, 1 mM EDTA and 1% glycerol and supplied to transaminase assay with L-Phe as the substrates and α -keto-glutalic acid as described below. For the PPA conversion, the powdered material (1.0 g) was homogenized in 0.1 M citrate buffer pH 6.0 containing 1% TritonX-100 in the presence of 5.7 g PVPP. After centrifugation (26,000 g, 15 min, 4 °C), 100 μ L the supernatant and 50 mM PPA (2.5 μ mol) in a 0.1 M citrate buffer pH 6.0 were incubated at 35 °C for 2 h and then ethyldecanoate in methanol (1.55 nmol) was added as an internal standard. The volatiles were extracted with 400 μ L of hexane:ethyl acetate (1:1, v/v). The organic layer was dried over Na₂SO₄ and subjected to GC-MS analyses. The GC-MS conditions are as shown in the paragraph 'GC-MS analysis'.

GC-MS analysis

Analyses of 2PE and PAld were performed using a GC-MS QP5050 (Shimadzu), which was controlled by a Class-5000 work station. For the analyses of 2PE, the GC was equipped with a capillary TC-WAX column (GL Sciences Inc., Japan) of 30 m $\times$ 0.25 mm I.D. and 0.25 μ m film thickness. For the analysis of PAld, the GC was equipped with a capillary TC-5 column (30 m $\times$ 0.25 mm I.D., 0.25 μ m film thickness, GL Sciences Inc., Japan).

For 2PE analysis, the column temperature was elevated from 60 °C (3 min hold) to 180 °C (40 °C/min) then to 240 °C (10 °C/min, 3 min hold). The injector temperature was 200 °C, the ionizing voltage was 70 eV, and the scanning speed was 0.5 scan/s with a range of m/z 76–200. For PAld analysis, the column temperature was elevated from 50 °C (3 min hold) to 90 °C (10 °C/min), then to 130 °C (30 °C/min) and then 290 °C (40

°C/min, 3 min hold). The injector temperature, the ionizing voltage, the scanning speed, and the m/z range were the same as for the 2PE analysis.

Substrate specificity of RyAAAT3

The reaction was initiated by the addition of the amino acid and performed at 45 °C under $V_{\max}$ conditions for L-Phe. The reaction was stopped by adding an equal volume acetonitrile after adding 200 nmol L-[²H₈]Phe as the internal standard. The sample was subjected to centrifugation (20,000 g , 10 min, 4 °C), filtered (Millex LH, Millipore) and subsequently analyzed by LC-MS (L, D-Phe, L-tyrosine, L-tryptophan) and an amino acid analyzer (Hitachi) (L-alanine and glycine). The products, PPA, 4-hydroxyphenylpyruvic acid and indole-3-pyruvic acid, were identified by comparing with the authentic specimens. The decrease in L-alanine and glycine were determined by amino acid analyzer following the manufacturing instructions.

Supplementary Table

Supplementary Table 1. Primers used in this study. A primer in QuantumRNA Universal 18S Internal Standard kit (Ambion) was used for 18srRNA.

Primers	sequence (5' - 3')
<u>3' RACE PCR</u>	
<i>RyAAAT1</i>	GCITAYCARGGITYGCIW
<i>RyAAAT2</i>	CTCGGACAGAAGCCTCTAAC
<i>RyAAAT3</i>	CCATGGAGAATGGAACCCATG
<u>5' RACE PCR</u>	
<i>RyAAAT1</i> for PCR	TGCTCCGGGTTCGATCCTG
<i>RyAAAT1</i> for nested PCR	TCAAACAGCTGATGTCGCA
<i>RyAAAT2</i> for PCR	CAGTTCGCAGTTTCTTCCAGG
<i>RyAAAT2</i> for nested PCR	GTGGAACCTGGAACCAGAACC
<u>Full length PCR</u>	
<i>RyAAAT1</i> Forward	ATGAACTCACTCTCCGCTTCC
<i>RyAAAT1</i> Reverse	TTAAGCAAGACGAGTAACAGC
<i>RyAAAT2</i> Forward	CACAGCAATCATGCCACCG
<i>RyAAAT2</i> Reverse	TTACATCCTTGAATAGCCTCTG
<u>Subcloning to pET28a</u>	
<i>RyAAAT1</i> Forward (BamH I)	GTGGGATCCATGAACTCACTCTCCGCTTC
<i>RyAAAT1</i> Reverse (Xho I)	ATACTCGAGAGCAAGACGAGTAACAGCTGC
<i>RyAAAT2</i> Forward (EcoR I)	GTGGAATTCATGCCACCGAAGGCATTGGAC
<i>RyAAAT2</i> Reverse (Sal I)	GTGGTCGACCATCCTTGAATAGCCTCTGTC
<i>RyAAAT3</i> Forward (Sal I)	GTGGTCGACGCATGGAGAATGGAACCCATGTG
<i>RyAAAT3</i> Reverse (Xho I)	GCGCTCGAGTAATTTTCTGGCATGCCTTTG
<u>Transcripts analysis</u>	
<i>RyAAAT3</i> transcripts analysis Forward	CACTGTGGGTCTTCCGCAAAC
<i>RyAAAT3</i> transcripts analysis Reverse	TCCCAAGGATGCTCGGAACTG
<u>dsRNA synthesis</u>	
<i>RyAAAT3</i> -N terminal promoter	T7 TAATACGACTCACTATAGGGATAAACCCCGGA AATCCTTG
<i>RyAAAT3</i> -N terminal	ATAAACCCCGGAAATCCTTG
<i>RyAAAT3</i> -C terminal	T7 TAATACGACTCACTATAGGGAATGGTTTATCCC

promoter	CAAAGGC
<i>RyAAAT3</i> -C terminal	AATGGTTTATCCCCAAAGGC

Supplementary Figures

RyAAAT3	1	MENGTHVNHQGGGGGVPVDT	TAA	TITIKGILSLL	LQNVD	--	EGESK	KR	ISLGMGDPTA	FS
CmAAAT	1	-----MEIGAVNSEMDT	ASTI	IKGILSLL	VQNAD	--	ENNGR	R	ISLGMGDPSAYS	
AtTAT	1	-----MENGATT	TTST	TITIKGILSLL	MESITTE	E	DEGGK	R	ISLGMGDPTLYS	
SpTAT1	1	---MEGNLVGKINN	QIEMET	PNNT	ITIKGIL	GLL	MANTEA	---	TDMK	KVISLGMGDPTLYS
PstTAT	1	--MEKGGKKWI	IRGNDK	LKVGTENT	I	RGL	LEV	MNS	NLN	--VNDERPI
										IFLGHGDPSPFT
RyAAAT3	59	CFHTTHIAQETVVDAIQ	SDKFNGY	APT	VGLPQ	TRRAIAEYLS	RDL	PYNLT	DDVFV	TSGC
CmAAAT	50	CFHTTRIAQDAVVD	CLESEK	FNGY	APT	VGLPQ	SRRAIAEYLS	RDL	PYKLT	DDVFV
AtTAT	48	CFRTTQVSLQAVSD	SLLSNK	FHGY	SPT	VGLPQ	ARRAIAEYLS	RDL	PYKLT	QDDVF
SpTAT1	55	CFHSPDVADAVIES	LTSHK	FNGY	SPT	VGLPQ	TRKAIADYLS	R	ELPEKL	CADDV
PstTAT	56	CFRTTHIVDDALNTA	IQSAKFNS	YP	PAG	TPARRSIAEH	LSRDL	PYKLT	STEDV	FLTSGC
RyAAAT3	119	TQAIDVALAMLARPGANILL	PRPGFFI	YELCS	AFRNLEVRH	L	DL	QDS	GWEV	NLDAVEAL
CmAAAT	110	TQAIDVALAMLARPGANILL	PRPGFFI	YELCSS	FQNL	LEVRH	FNLL	PQ	QGW	VDLHAIETL
AtTAT	108	TQAIDVALSMLARPRANILL	PRPGFFI	YELCAK	FRHLEVR	YV	DL	P	ENGWE	IDLDAVEAL
SpTAT1	115	TQAIETALSILARPGANILL	PRPGFFI	YALCAA	FRNIEVR	Y	FDL	P	DKG	WEVDLNAVEAL
PstTAT	116	RQAIETITTVLACPGGNIL	IPKPG	Y	PDACAV	FHNLEVR	HFDLL	PEKA	WEVD	LDLDAVEAL
RyAAAT3	179	ADQNTVAMVIINPGN	PCGNVSYQHL	SKIAETAK	KLRL	IL	VI	AD	EVYGH	LAFGDKPFVPMG
CmAAAT	170	ADKNTVALVIINPGN	PCGNVSYQHL	SKIAETA	EKL	GIL	VI	AD	EVYGH	LAFGSRPFVPMG
AtTAT	168	ADENTVALVVIINPGN	PCGNVSYQHL	MKIAE	S	AKKL	G	FL	VI	AD
SpTAT1	175	ADHNTIGIVVIINPGN	PCGNVSYQHL	QOIAETAK	KLRTI	VI	AD	EVYGH	LAFGAN	PFVPMG
PstTAT	176	ADENTVALVIINPGN	PCGNVYTYEHL	KKVAETAK	R	L	GIP	VI	AD	EVY
										AHLIFGSNPFVPMG
RyAAAT3	239	VFGSTVPVLT	LGSLSKRWIVPGW	R	L	GWFVT	TD	PCGM	FRKPKV	IERIKKYFDILGGPATFI
CmAAAT	230	VFGSTVPVLT	LGSLSKRWIVPGW	R	L	GWFVT	SD	PSGM	FRKPKV	IERIKKYFDILGGPATFI
AtTAT	228	VFGSTVPVLT	LGSLSKRWIVPGW	R	L	GWFVT	TD	PSGS	F	KDPKIIERIKKYFDILGGPATFI
SpTAT1	235	IFGDIAPVLT	LGSLSKRWIVPGW	R	L	GWFVT	ND	PN	GTFKNPKF	VERIKKYCDICGGPATFI
PstTAT	236	VFGSTVPVLT	LGSLSKRWIVPG	R	L	GWFVT	TD	PSG	FLK	DEIVSLIKQCLNMSTSPACVI
RyAAAT3	299	QAAVPSILGKTEB	VFFKKTIYLLKQ	SSDICS	DKIKDIP	CL	TC	PNK	PEGSMA	V
CmAAAT	290	QAAVPRILEST	DEVFFKKTI	INILKQ	TS	EICCR	KIKEIP	CITCTH	REGSMA	MVRLNIDL
AtTAT	288	QAAVPTILEQ	TDESFFKKTL	NSLKN	SSDICC	DMIKEIP	CIDSS	HR	REGSMA	MVRLNIDL
SpTAT1	295	QAAVPRILEQ	TEDVFFRKT	VNLLKWT	ADICCD	KIKEIP	CIS	CPYK	PEGSMA	V
PstTAT	296	QGAIPQILENT	KEDEFFENI	ISLLCQA	IDIC	YEEIKEI	ACITLL	PR	EGSM	FLMVKLNTVL
RyAAAT3	359	LEDISDDIDFCFKLAKEES	VILP	LGTA	VGLKNWIR	ITFAAD	PPS	LEEAF	FRRTK	CFYQRHA
CmAAAT	350	LEDISDDIDFCFKLAKEES	LVILP	LGTA	VGLKNWLR	ITFAV	DP	S	FL	EEALGR
AtTAT	348	LEDISDDIDFCFKLAKEES	VILP	LGTA	VGLKNWLR	ITFAAD	ATS	EEAF	FR	KIKCFYLRHA
SpTAT1	355	MIDISDDIDFCFKLAKEES	VILP	LGTA	VGLKNWIR	ITFAAD	PPAS	LEEAL	GR	LKSFCQRHS
PstTAT	356	LEDISDDIDFCFKLAKEES	VILP	LGTA	VGLKNWLR	ITFSID	LAS	LK	DGLQRM	KMFCCRHA
RyAAAT3	419	RKL----								
CmAAAT	410	LML----								
AtTAT	408	KTQYPTI								
SpTAT1	415	YQONDHH								
PstTAT	416	KQD----								

Supplementary Fig. 1. Amino acid sequence multiple alignments of RyAAAT3 and plant AAAT/TyrAT. *Cucumis melo* L. AAAT (CmAAAT, ADC45389), *Papaver somniferum* TyrAT (PsTAT, ADC33123), *Solanum pennellii* TyrAT 1 (SpTAT, ADZ24702) and *Arabidopsis thaliana* TyrAt (AtTAT, NP_200208). Black arrowheads indicate the conserved Lys residues that covalently bind the PLP cofactor and white arrowheads indicate conserved amino acids proposed to possess crucial roles in catalysis (Blankenfeldt et al., 1999; Yennawar et al., 2001). Underline shows

64 aminotransferases family-I PLP attachment site. Black dots indicate *N*-glycosylation
65 site predicted in Genetyx version 8.0 software.
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Supplementary Fig. 2. Comparison of transaminase activity among RyAAAT1-3. Selected ion traces at m/z 163 $[M-H]^-$ on LC-MS analyses of L-Phe metabolites produced with recombinant RyAAAT1-3 extracted from *E. coli* cultures. The asterisks depict peaks for authentic PPA and PPA detected in each respective chromatogram. PPA production was not detectable in RyAAAT1 and 2 reaction mixtures after 10 min reaction time.

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Supplementary Fig. 3. Characterization of RyAAAT3 enzymatic parameters. (A) Optimum pH; (B) Optimum temperature of the transaminase reaction producing PPA from L-Phe with α -ketoglutaric acid as an amino acceptor. (mean of 3 replicates $\pm$ SD).

Supplementary Fig. 4. LC-MS chromatogram of PPA conversion to L-Phe, reverse reaction of RyAAAT3. RyAAAT3 transaminates PPA with L-glutamic acid as an amino donor. Negative control stands for beginning of the reaction. The asterisk depicts peak for authentic L-Phe and that detected in the reaction mixtures. Retention time of L-Phe was 5.98 min. Reaction mixtures (150 μ L) containing 10 mM PPA, 10 mM L-glutamic acid, and 30 μ L enzyme solution in a 0.5 M Tris-HCl buffer (pH 9.0) was incubated at 45 $^{\circ}$ C for 10 min. The reaction was stopped by adding equal volumes acetonitrile after adding 200 nmol L-[2 H $_8$]Phe as an internal standard. After the sample was centrifuged (20,000g, 10 min, 4 $^{\circ}$ C), the supernatant was filtered (Millex LH, Millipore) and analyzed by LC-MS.

Supplementary Fig. 5. A phylogenetic tree of members of RyAAATs and AspATs, AlaATs, TrpATs, TyrATs and SUPERROOT 1 are from various plants. The multiple alignments of AAATs were performed with ClustalW 1.81. (Abbreviations are as follows: At, *Arabidopsis thaliana*; Cm, *Cucumis melo* (melon); Gm, *Glycine max*; Ms, *Medicago sativa*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Pf, *Perilla frutescens*; Ps, *Papaver somniferum*; Ry, *Rosa 'Yves Piaget'*; Sm, *Salvia miltiorrhiza*; Sp, *Solanum pennellii* (tomato); Ss, *Solenostemon scutellarioides*; Ta, *Triticum aestivum*; Zm, *Zea mays*; BCAT, branched chain aminotransferase; SUR1, SUPERROOT 1 (similar to TyrAT). A molecular phylogenetic tree was constructed by the neighbor-joining (NJ) method. The statistical significance of the NJ tree topology was evaluated by bootstrap analysis 1,000 iterative tree construction. The tree was drawn with CLC Sequence Viewer 6. Bootstrap values (1000 replicates) are given for the nodes. Bootstrap values over 800 are shown. All listed aminotransferases are as follows: AtTrpAT, NP177213; AtTAT, NP200208; GmTAT, AAY21813; MtTAT, AAY85183; SpTAT1, ADZ24702; SmTAT, ABC60050; PfTAT, ADO17550; SsTAT, CAD30341; PsTAT, ADC33123; AtSUR1, Q9SIV0; CmAAAT, ADC45389; CmBCAT, ADC45390; GmAspAT, AAC50015; MsAspAT, CAA43779; AtAspAT, NP196713; TaAspAT, ABY58643; GmAlaAT, ABW17197; AtAlaAT, NP177215; OsAlaAT, AAO84040; ZmAlaAT,

AAC62456.

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