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ABSTRACT

trans-Cinnamic acid (CA) is a precursor of many phenylpropanoid compounds, including catechins and aroma compounds, in tea (*Camellia sinensis*) leaves, and is derived from L-phenylalanine (L-Phe) deamination. We have discovered an alternative CA formation pathway from L-Phe *via* phenylpyruvic acid (PPA) and phenyllactic acid (PAA) in tea leaves through stable isotope-labeled precursor tracing and enzyme reaction evidence. Both PPA reductase genes (*CsPPARs*) involved in the PPA-to-PAA pathway were isolated from tea leaves and functionally characterized *in vitro* and *in vivo*. CsPPAR1 and CsPPAR2 transformed PPA into PAA and were both localized in the leaf cell cytoplasm. *Rosa hybrida* flowers (economic crop flower), *Lycopersicon esculentum* Mill fruits (economic crop fruit), and *Arabidopsis thaliana* leaves (leaf model plant) also contained this alternative CA formation pathway, suggesting that it occurred in most plants, regardless of different tissues and species. These results improve our understanding of CA biosynthesis in tea plants and other plants.

KEYWORDS: Tea; *Camellia sinensis*; *trans*-Cinnamic acid; L-Phenylalanine; Phenylpyruvic acid; Phenyllactic acid; Phenylpyruvic acid reductase

INTRODUCTION

Tea is an aqueous infusion of processed leaves from the plant *Camellia sinensis* (L.) O. Kuntze, which is the second most popular beverage globally next to water owing to its characteristic functions and quality. Many specialized secondary metabolites derived from L-phenylalanine (L-Phe) contribute to tea function and quality. Catechins, accounting for 12%–24% of dried tea leaves by weight, are considered natural antioxidants with dietary importance for human health.[1,2] Catechins are also responsible for the taste and color of tea, which affect tea quality.[3] Volatile phenylpropanoids/benzenoids (VPBs) are another class of L-Phe-derived metabolites and the second most ubiquitous volatile class in plants.[4] In tea, VPBs can impact tea quality through contributions to tea aroma and odor.[3] Therefore, the metabolism of L-Phe plays an important role in tea quality and function, and deserves further investigation. Recently, catechins and some characteristic VPBs have been directly investigated in tea leaves, containing the biosynthetic pathways involved in their production, enzymes that catalyze these pathways, genes that encode these enzymes, and the responses to abiotic and biotic stresses on the formation of these metabolites in tea leaves.[5–10] Among these studies, most have focused on the final biosynthetic steps of catechins and VPBs, such as functional characterization of the related genes and enzymes. However, upstream pathway information on tea is very limited, with most reports referring to other plants.

Recently, much research has focused on the upstream pathway of L-Phe-derived metabolites in various plants, resulting in useful and insightful discoveries, including determination of the role of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase in the shikimate pathway in *Arabidopsis*,[11] the participation of *Chorismate mutase 1* in VPB formations in *Petunia* flowers,[12]

the synthesis of L-Phe from prephenate in *Petunia* flowers and from phenylpyruvic acid (PPA) in plants,[13–15] and the role of CoA ligases in benzenoid biosynthesis in *Petunia* flowers.[16] Furthermore, some studies have investigated the synthesis of *trans*-cinnamic acid (CA) derived from L-Phe owing to its important roles. In plants, although CA is involved in plant metabolism and physiology, its biosynthesis remains unclear. The only pathway to CA formation in plants that is generally accepted is the deamination of L-Phe under the action of phenylalanine ammonia-lyase (PAL).[17] In some microorganisms, such as *Propionibacterium freudenreichii*, L-Phe can be transaminated to PPA, which is then further transformed into phenyllactic acid (PAA).[18] Related pathways have also been confirmed in some plant species.[6,19] Previous review has proposed that there are two candidate pathways of CA formation by the dehydration of α-hydroxy acid or removing the element of ammonia from α-amino acid.[20] In addition, it remains to distinguish between these two possible routes in the previous tracer study.[20] This led us to consider whether an alternative pathway for CA formation from L-Phe *via* PPA and PAA occurs in plants, such as tea.

This study aimed to investigate the occurrence of an alternative pathway to CA in tea leaves. Accordingly, L-[$^2H_8$]Phe (stable isotope-labeled precursor) was applied to determine the occurrence of CA biochemical pathway(s) in tea leaves. The chemical synthesis of [$^2H_6$]CA (stable isotope-labeled compound) was used as an authentic standard for the L-[$^2H_8$]Phe-derived CA product. Furthermore, two phenylpyruvic acid reductase genes (*CsPPARs*) involved in the pathway from PPA to PAA were also functionally identified. We also chemically synthesized [$^{13}C_6$]PAA for tracing in tea leaves to verify the pathway from PAA to CA. Finally, other plants, including *Rosa hybrida* flowers (economic crop flower), *Lycopersicon esculentum* Mill fruits (economic crop fruit), and *Arabidopsis thaliana* leaves (leaf model plant), were also investigated

to confirm whether the identified alternative pathway to CA was specific to tea leaves or ubiquitous in most plants. This study aimed to discover an alternative pathway for CA formation from L-Phe in plants, and enhance our understanding of L-Phe metabolism and CA biosynthesis.

Chemical reagents

CA was obtained from Wako Pure Chemical Industries Ltd., Japan. L-[$^{2}H_{8}$]Phe, L-[$^{13}C_{6}$]Phe, [$^{2}H_{6}$]aldehyde and [$^{2}H_{7}$]CA were obtained from Cambridge Isotope Laboratories Inc., Cambridge, MA, USA. *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Regis Chemical Co., Morton Grove, Illinois, USA. [$^{2}H_{6}$]CA (purity, ≥95%) and [$^{13}C_{6}$]PAA (purity, ≥95%) were synthesized in our lab.

Plant materials and growth conditions

In this study, the tea sample was obtained from cultivar 'Jinxuan' grown in Yingde town, Guangdong province, China. Tea cultivar 'Jinxuan' is mainly grown in southern China. *R. hybrida* 'Tineke' flower and *L. esculentum* Mill fruit were purchased from the market. *A. thaliana* was grown under set conditions (22±2 °C, 70±2% relative humidity, photoperiod of 8 h dark/16 h light).

Plants treatment with L-[$^{2}H_{8}$]Phe

Plant treatments with L-[$^{2}H_{8}$]Phe were performed as reported in a previous study.[21,22] *C. sinensis* 'Jinxuan' branches with one bud and three leaves collected in March 2019 were cultivated in two different solutions (Figure S1A), namely, H_2O and 12 mM L-[$^{2}H_{8}$]Phe. In the previous study,[21] the times for feeding L-[$^{2}H_{8}$]Phe into 'Jinxuan' branches were set on 3 and 7 d,

and the labelled L-Phe-derived metabolites could be detected on 3 d. Therefore, in the study, the feeding time was only set on 3 d for *C. sinensis*. *R. hybrida* 'Tineke' flowers were purchased from the market in March 2019. Petals were separated from *R. hybrida* 'Tineke' flower, and the bases were immersed in two different solutions (Figure S1B), namely, H_2O and 12 mM L-[$^2H_8$]Phe. Former study has confirmed that labelled L-Phe-derived metabolites could be detected on 1.5 d in *R. hybrida* 'Tineke' flower when fed with L-[$^2H_8$]Phe.[22] To obtain more labelled L-Phe-derived metabolites, the feeding time was set on 2 d for *R. hybrida* 'Tineke'. *L. esculentum* Mill fruit was purchased on the market in March 2019. The fruit of *L. esculentum* Mill was treated with two different solutions (Figure S1C), namely, H_2O and 12 mM L-[$^2H_8$]Phe. Every fruit was treated with solutions (300 μL) every 12 h (total of 14.4 μmol for L-[$^2H_8$]Phe). Former study has confirmed that labelled L-Phe-derived metabolites could be detected on 1 d when fed with L-[$^2H_8$]Phe in *L. esculentum* Mill fruit.[21] After 1 d-feeding, the tomato fruit was in a good state and could be kept for longer periods. In addition, to obtain more labelled L-Phe-derived metabolites, the feeding time was set on 2 d for *L. esculentum* Mill. *A. thaliana* (4–5 weeks old) cultivated in the laboratory was used in the stable isotope tracing experiment. *A. thaliana* leaves were treated with two different solutions (Figure S1D), namely, H_2O and 12 mM L-[$^2H_8$]Phe. Former study has confirmed that labelled Phe-derived metabolites could be detected on 18 h when fed with L-[$^2H_8$]Phe in *A. thaliana* leaves.[23] After 18 h-feeding, the *A. thaliana* plants were in a good state and could be kept for longer period. In addition, to obtain more labelled L-Phe-derived metabolites, the feeding time was set on 1 d for *A. thaliana*. Feeding time of 3 days was too long for cut *R. hybrida* 'Tineke' flower, postharvest *L. esculentum* Mill fruit and soil-free *A. thaliana* leaves. Therefore, the sample treatments were conducted under a set conditions of 70% humidity, 25 °C, and a photoperiod of 8 h dark/16 h light for several days (3

days for *C. sinensis*, 2 days for *R. hybrida* 'Tineke' and *L. esculentum* Mill, and 1 day for *A. thaliana*). L-[$^2H_8$]Phe entered the tea leaves and *R. hybrida* 'Tineke' flower *via* the cut stem, and entered *A. thaliana* leaves *via* root driven by the transpiration stream.

Chemical synthesis of [$^2H_6$]CA

The synthesis of [$^2H_6$]CA was performed according to a previously reported method.[24] [$^2H_6$]Aldehyde (560 mg, 5.0 mmol) and malonic acid (520 mg, 5.0 mmol) were added to pyridine solution (6 mL) followed by a catalytic amount of morpholine (44 mg, 44 μL, 0.5 mmol). The reaction was conducted by slowly heating to 110 °C and maintaining this temperature for 6 h. Thin-layer chromatography (TLC) was used to monitor the reaction. The reaction was quenched into water (30 mL) when cooled to room temperature. After filtration, the precipitated solid was washed with water (5 mL) two times. The product was then dried by suction filtration using a Buchner funnel for 15–60 min. The resulting solid product was dried in an oven at 50–60 °C overnight. The yield of [$^2H_6$]CA was 85% (654 mg).

As-obtained [$^2H_6$]CA was a white solid with more than 95% purity. The purity of [$^2H_6$]CA was determined based on its NMR spectra (Figure S2A). ^{1}H NMR (500 MHz, CD_3OD, ppm) δ_H 6.49 (s, 1H); ^{13}C NMR (125 MHz, CD_3COCD_3, ppm) δ_C 117.2, 127.8, 128.0, 128.1, 128.3, 128.5, 128.7, 130.1, 130.3, 130.4, 133.8, 146.6, 146.7, 146.9, 172.5; HRESIMS (*m/z*) calcd. for $C_{31}H_{35}O_6$ $[M–H]^-$ 503.2439, found 503.2436.

Determination of labeled CA

Identification and analysis of [$^2H_7$]CA and [$^2H_6$]CA were using gas chromatography mass spectrometer (GC–MS) combined with MSTFA derivatization according to the previous study

with some modification.[25] Finely powdered samples (500 mg for *C. sinensis* leaves and *A. thaliana* leaves, fresh weight) were extracted in cold methanol (4 mL) by ultrasonic extraction for 20 min. The extract was purified in chloroform (4 mL) and water (1.6 mL). The labelled metabolites of *L. esculentum* Mill Fruit and *R. hybrida* 'Tineke' flower (2 g, fresh weight) were extracted using 8 mL of cold methanol, and partially purified using 8 mL of cold chloroform and 3.2 mL of cold water with the above extraction method. After the extraction and preliminary purification, all samples were subjected to centrifugation (10,000 *g*, 4 °C, 10 min), and the supernatants (4 mL for *C. sinensis* leaves and *A. thaliana* leaves, and 9 mL for *L. esculentum* Mill Fruit and *R. hybrida* 'Tineke' flower) were collected. The supernatants (methanol) were dried under gaseous N_2, and the resulting solution (only water) was diluted to 2 mL using water. The extract in water was added with 48 μL hydrochloric acid (0.25 M) and extracted twice with ethyl acetate (2 mL). The ethyl acetate phase was collected and dried under gaseous N_2. The dry power was derivatized with MSTFA (70 μL) at 37 °C for 60 min and then centrifuged. The MSTFA derivates (1 μL) were then subjected to gas chromatography mass spectrometer (GC–MS) analysis carried on a GC–MS QP2010 SE (Shimadzu Corporation, Kyoto, Japan) equipped with GCMS Solution software (Ver. 2.72. Samples were injected into the injection port of GC under 230 °C temperature condition for 1 min in splitless mode. Labelled metabolites were separated on an HP-5 column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, California, USA). As the carrier gas, helium was in a velocity of 1 mL/min. The temperature of GC oven was initially set at 100 °C for 5 min, ramped up to 300 °C at a rate of 10 °C/min, and then kept at 300 °C for 15 min. Mass spectrometry was operated in a full scan mode (mass range, *m/z* 40−350), and a selected ion mode (*m/z* 138, 212 and 227 for $[^2H_7]$CA; *m/z* 137, 211 and 226 for $[^2H_6]$CA).

155

156 **Chemical synthesis of [$^{13}C_6$]PAA**

157 [$^{13}C_6$]PAA was prepared according to a previously reported method.[26] L-[$^{13}C_6$]Phe (171 mg,
158 1.0 mmol) was dissolved using water (3.0 mL), and $NaNO_2$ (412 mg, 6.0 mmol) was added in
159 one portion, followed by the addition of concentrated H_2SO_4 (105 μL) at 0 °C. The mixture was
160 then stirred under a N_2 atmosphere at room temperature for 12 h. The reaction solution was
161 extracted using EtOAc (5 mL) five times. The obtained EtOAc phases were combined, dried by
162 anhydrous Na_2SO_4, and filtered through a Buchner funnel. Solvent was removed from the
163 resulting solution by rotary evaporation to give the product as a slightly yellow solid. The
164 product was further washed with hexane to give pure product [$^{13}C_6$]PAA in 78% yield (129 mg,
165 0.78 mmol).

166 As-obtained [$^{13}C_6$]PAA was a white solid with more than 95% purity. The purity of
167 [$^{13}C_6$]PAA was determined based on its NMR spectra (Figure S2B). ^{1}H NMR (500 MHz,
168 CD_3OD, ppm) δ_H 2.93 (m, 1H), 3.14 (m, 1H), 4.41 (m, 1H), 7.05–7.47 (m, 5H); HRESIMS (*m/z*)
169 calcd. for $C_{31}H_{35}O_6$ $[M–H]^-$ 503.2439, found 503.2436.

170

171 **Recombinant expression of *CsPPARs* and their enzyme assay**

172 Recombinant expression of *CsPPARs* was performed according to previous studies.[6,22] PCR
173 was used to obtain the full length open reading frames (ORFs) of *CsPPAR1* and *CsPPAR2*. The
174 primers are shown in Table S1. Details of *CsPPARs* recombinant expression are provided in the
175 supporting information. After recombinant expression, the *Escherichia coli*-expressed proteins
176 were subjected to SDS-PAGE and western-blot analysis (the related description also provided in
177 supporting information). The identified recombinant proteins were used in the enzyme assay and

substrate selectivity analysis according to a previously reported method.[19] The reaction mixture (500 μL) contained 2 mM PAA or *p*-hydroxyphenylpyruvic acid (*p*-HPPA) as substrate, 2 mM NADPH in potassium phosphate (pH 8.0, 50 mM), and semi-purified protein (25 μg). Empty vector (pET32a) enzyme was used as a negative control. As PPA and *p*-HPPA were almost water-insoluble, methanol was added to achieve dissolution, resulting in a reaction mixture containing 1% methanol. The temperature and time of reaction was 30 °C and 2 h, respectively. The reaction solution (500 μL) was mixed using 0.5 M hydrochloric acid (20 μL). The reaction mixture was then extracted twice with ethyl acetate (1 mL). The extract was dried by blowing with nitrogen, and the residue was redissolved in acetonitrile (200 μL). After filtering through a 0.22-μm membrane, the catalytic products (100-fold diluted for CsPPAR2) were analyzed by UPLC–QTOF MS (ACQUITY UPLC I-Class/Xevo G2-XS QTOF, Waters Corp., Milford, MA, USA). Compound identification was conducted using the retention time (t_R) and mass data (PAA, t_R = 3.41 min, *m/z* 165.0552 $[M–H]^–$; 4-hydroxyphenyllactic acid, t_R = 2.02 min, *m/z* 181.0501 $[M–H]^–$). Details of UPLC–QTOF MS analysis are provided in our previous study and the supporting information.[6]

Identification of function of CsPPAR in *Nicotiana benthamiana* overexpression lines

Analysis of CsPPAR activity in *N. benthamiana* overexpression lines was conducted according to previous studies.[6,22] The full-length ORFs were subcloned into pCAMBIA3300 vector to obtain expression constructs (pCAMBIA3300-CsPPAR1 and pCAMBIA3300-CsPPAR2) using the primers listed in Table S1. Details of the vector constructions and transformation of recombinant vector into *N. benthamiana* leaves are provided in the supporting information. After 5 days of infiltration, the tobacco leaves were plucked and

each leaf was placed in a 1.5-mL tube containing 3 mM sodium phenylpyruvate dissolved in distilled water (1 mL), and water alone as control. All samples were subjected to an 8 h dark/16 h light photoperiod at 25 °C in an incubator. After 24 h, 500 μL of the phenylpyruvate solution was absorbed by the tobacco leaf in each treatment, and the leaves were harvested and stored at −80 °C for metabolite analysis. As the PAA content in *N. benthamiana* overexpression lines was below the detection limit of UPLC−QTOF MS, GC–MS analysis combined with MSTFA derivatization was used to investigate changes in the PAA content in *N. benthamiana* leaves after transient overexpression of CsPPARs, as shown in the supporting information.

Subcellular localization of *CsPPARs* in transient transgenic leaves of *N. benthamiama*

Leaves of *CsPPARs*-overexpressed *N. benthamiama* were cut into pieces (nearly 1-2 cm^2), and observed under confocal laser microscope (Zeiss LSM 510 META, Carl Zeiss, Jena, Germany). For GFP fluorescence observation, the images of confocal microscope were taken with a 40× water objective under 488 nm excitation wavelength.

Subcellular localization of *CsPPARs* in protoplasts of *Arabidopsis* leaves

To investigate the subcellular localization of CsPPARs, the full-length ORFs were subcloned into pSAT6-EYFP-N1 vector to obtain expression constructs (pSAT6-CsPPAR1 and pSAT6-CsPPAR2) using the primers listed in Table S1. The protoplasts from *Arabidopsis* leaves were prepared according to a previous report.[27,28] Nearly 3‑4 weeks old *A. thaliana* plants were applied to protoplast isolation. The lower epidermal surface cell layer was peeled away, and the peeled leaves were placed on a Petri dish containing the enzyme solution (total volume, 10 mL; comprising 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 5.7), 0.3% macerozyme R10,

20 mM KCl, 1.5% cellulase R10, 0.4 M mannitol, and 10 mM $CaCl_2$). After allowing to stand for 1 h, protoplasts were released into the solution. According to a previously reported method,[28] the constructed expression vectors were introduced into protoplast cells from *Arabidopsis*. The protoplasts were centrifuged (100 ×*g*, 2 min) and then washed twice with cold W5 solution (2 mM MES (pH 5.7) comprising 154 mM NaCl, 5 mM KCl and 125 mM $CaCl_2$). Finally, the protoplasts were resuspended in an MMG solution (4 mM MES (pH 5.7) comprising 0.4 M mannitol and 15 mM $MgCl_2$). pSAT6-CsPPARs plasmid DNA (10 μL, 10–20 μg of plasmid DNA) and polyethylene glycol 4000/Ca (PEG/Ca) solution (110 μL) were added to the protoplasts (100 μL, equivalent to 2×10^4 protoplasts). The mixture was incubated at room temperature for 15 min and then diluted with W5 solution (400–440 μL). The diluted solution was centrifuged at 100 ×*g* for 2 min. The resulting pelleted protoplasts were re-suspended in W5 solution (100 μL) and incubated at 23 °C for 12–16 h. A Zeiss LSM 510 META confocal laser microscope was used to observe the protoplasts using a 40× water objective in multitrack channel mode. The excitation wavelengths and emission filters were 488 nm/band-pass 505–530 nm for yellow fluorescent protein (YFP), and 488 nm/band-pass 650–710 nm for chloroplast autofluorescence. LSM 510 version 4.2 software (Zeiss) was used to perform image processing.

Feeding experiment using [$^{13}C_6$]PAA in tea leaves and determination of [$^{13}C_6$]CA

To confirm the role of PAA in CA synthesis in tea leaves, the tea leaves were supplemented with [$^{13}C_6$]PAA. *C. sinensis* 'Jinxuan' branches collected in May 2019 were cultivated in two different solutions (Figure S1A), namely, H_2O (control) and 6 mM [$^{13}C_6$]PAA ([$^{13}C_6$]PAA treatment). The samples were treated under set conditions of 70% humidity, an 8 h dark/16 h light photoperiod, and 25 °C for 2 days. The method used for [$^{13}C_6$]CA determination was the

same as that used for [$^2H_6$]CA identification.

Statistical analysis

One-way analysis of variance (ANOVA) was applied to determine the differences among three groups and a two-tailed Student's *t*-test was used to determine the differences between two groups.

RESULTS AND DISCUSSION

There are two pathways leading from L-Phe to CA in tea leaves

The deamination of L-Phe under action of PAL (Figure 1, pathway I) is widely considered the only pathway to CA formation in plants. To investigate whether an alternative pathway for CA formation from L-Phe occurs in plants (Figure 1, pathway II), tea leaves, were supplemented with L-[$^2H_8$]Phe.[1,29] First, we obtained authentic standards of [$^2H_6$]CA (Figure 2A) through chemical synthesis and [$^2H_7$]CA commercially. Using these authentic standards, both [$^2H_6$]CA and [$^2H_7$]CA were identified in tea leaves supplemented with L-[$^2H_8$]Phe (Figures 2B and 2C). This suggested that an alternative pathway for CA formation from L-Phe occurred in tea leaves. Relative contribution of two pathways leading to CA have been calculated by [$^2H_6$] and [$^2H_7$] concentrations based on the peak areas of their MSTFA-derivates, and the ratio of [$^2H_6$]-pathway to [$^2H_7$]-pathway is nearly 1.32. Previous studies have indicated that another biosynthetic pathway for CA from L-Phe exists, proceeding *via* PPA and PAA.[18,20] The present study showed that this alternative pathway to CA from L-Phe (Figure 1, pathway II) indeed occurred not only in microorganisms, but also in some plants, such as tea leaves. Stable isotope-labeled precursor

L-[$^2H_8$]Phe was used to trace the pathway in tea leaves and investigate CA products with different numbers and positions of ^2H, to propose the potential multiple pathways (Figure 1).

Using L-[$^2H_8$]Phe tracing in plants, multiple pathways from L-Phe to its downstream volatile products, such as 2-phenylethanol, have been proposed in tea leaves, rose flowers, petunia flowers, and tomato fruits.[21,22,30–32] Three biosynthetic pathways have been discovered to 2-phenylethanol from L-Phe in plants.[21] Furthermore, using L-[$^2H_8$]Phe tracing in flowers of tea plants, two potential pathways from L-Phe to its downstream volatile products, such as acetophenone and 1-phenylethanol, have been proposed.[25] Furthermore, based on L-[$^2H_8$]Phe tracing investigations in plants, the formation of acetophenone from L-Phe in plants other than flowers of tea plants was small, resulting in little accumulation of 1-phenylethanol in these other plants.[23,33] In this study, using L-[$^2H_8$]Phe tracing in tea leaves, an alternative pathway for CA formation from L-Phe was identified (Figure 2). As a successful transgenic system has not be established for tea plants, stable isotope-labeled precursor L-[$^2H_8$]Phe tracing in tea plants might be a suitable approach to discovering unknown L-Phe-derived metabolic pathways through *in vivo* evidence.

CsPPAR1 can convert PPA to PAA, which is a precursor of CA in tea leaves

According to the position of ^2H substituents in [$^2H_6$]CA, we proposed that [$^2H_7$]PPA and [$^2H_7$]PAA were the key intermediate metabolites in the pathway from L-[$^2H_8$]Phe to [$^2H_6$]CA (Figure 1, pathway II). The pathway to PPA from L-Phe has been validated and the involved enzyme, AAAT, has been functionally identified in tea leaves and other plants, such as rose flowers and melon.[6,22,30,34] Therefore, we further investigated the pathway from PPA to PAA. Two enzymes (PPA reductases, PPARs) were functionally identified in tea leaves. A comparison

between homologous sequences of *PPAR* in tea leaves and a reported *AbPPAR* (which has been validated to be involved in the pathway from PPA to PAA) from *Atropa belladonna* was performed.[19] By searching against the Transcriptome Shotgun Assembly (TSA) database, CsPPAR1 and CsPPAR2 were found, showing 52.48% and 43.15% similarity with *AbPPAR*, respectively (Figure S3). The CsPPAR1 and CsPPAR2 recombinant proteins produced in *E. coli* (Figures 3A and 3B) were able to transform PPA into PAA *in vitro*, while CsPPAR2 preferred *p*-HPPA as substrate compared with PPA (Figure 3C). Functional identification in *N. benthamiana* plants showed that CsPPAR1 and CsPPAR2 both converted PPA into PAA in plants (Figure 4). CsPPAR1 and CsPPAR2 were located in the cytoplasm using *N. benthamiana* leaf system (Figure 5A). In addition, the subcellular location of CsPPARs was used to confirm once again based on co-localization of maker protein in the *A. thaliana* leaf protoplast system, and the result was the same with the one obtained using *N. benthamiana* leaf system (Figure 5B). To further confirm the involvement of PAA in CA formation, [$^{13}C_6$]PAA authentic standard was chemically synthesized (Figure 6A) and supplied to tea leaves. More [$^{13}C_6$]CA was detected in [$^{13}C_6$]PAA treatment leaves compared with the control (Figure 6B), suggesting that PAA was converted into CA in tea leaves. Overall, these results indicated that an alternative pathway to CA derived from L-Phe *via* PPA and PAA occurred in tea leaves.

The pathway from L-Phe to PPA has been confirmed in tea leaves, melon, rose flowers, petunia flowers, and tomato fruits when studying 2-phenylethanol pathways in these plants, as the pathway from L-Phe to PPA is the alternative upstream pathway of 2-phenylethanol.[21,22,30–32,34] Furthermore, the pathway involving enzyme AAAT was identified and functionally characterized in melon, rose flowers, and tea leaves.[6,22,30,34] In contrast to the pathway from L-Phe to PPA, very little information is available for the pathway from PPA to

316 PAA. We chemically synthesized [$^2H_7$]PPA, supplied it to the tea leaves, and attempted to detect
317 the formation of product [$^2H_7$]PAA in tea leaves. As PPA is unstable, the synthesized [$^2H_7$]PPA
318 compound contained [$^2H_7$]PAA. Furthermore, [$^2H_7$]PPA readily autotransformed into [$^2H_7$]PAA,
319 even in sodium bicarbonate buffer (pH 8). Therefore, [$^2H_7$]PPA tracing in tea leaves failed to
320 elucidate the pathway from PPA to PAA in tea leaves. Until now, the PPARs involved in the
321 pathway from PPA to PAA have been characterized in *Wickerhamia fluorescens* TK1,
322 *Lactobacillus* sp. CGMCC9967 and *Atropa belladonna*.[19,35,36] In *Atropa belladonna*, AbPPAR is
323 the key upstream enzyme determining the biosynthesis of tropane alkaloids that are active
324 compounds, because suppression of *AbPPAR* disrupted tropane alkaloid biosynthesis.[19]
325 Biochemically, PPAR may catalyze the similar type of reaction to hydroxyphenylpyruvate
326 reductases (HPPR). Because PPAR has a hydroxyl acid dehydrogenase domain, which also
327 exists in formate/glycerate dehydrogenase like family.[19] HPPR belongs to this family and is able
328 to catalyze *p*-HPPA into *p*-hydroxyphenyllactic acid (*p*-HPLA). In substrate selectivity analysis,
329 *p*-HPPA was used as a substrate to determine the function of *E. coli*-expressed AbPPAR *in*
330 *vitro*,[19] which was also carried out in the functional characterization of CsPPARs. In the present
331 study, CsPPARs from tea leaves were also shown to catalyze the reduction of PPA to PAA both
332 *in vitro* (*E. coli* system) and *in vivo* (*N. benthamiana* overexpression system) (Figures 3 and 4).
333 These results suggested that PPAR was present not only in microorganisms, but also in some
334 plants. As a successful transgenic system has yet to be established for tea plants, overexpression
335 or RNAi of *CsPPARs* in tea leaves cannot be performed at present. In future, direct *in vivo*
336 evidence of the involvement of PPAR in CA biosynthesis in tea plants needs to be obtained.
337 Compared with the pathway from PPA to PAA, almost no confirmed information on the pathway
338 from PPA to CA is available in plants. In the present study, [$^{13}C_6$]PAA was investigated to

339 determine whether it could be transformed into [$^{13}C_6$]CA in tea leaves. Similar to PPA, PAA was
340 unstable and the [$^{13}C_6$]PAA standard contained [$^{13}C_6$]CA (Figure 6B). However, after
341 supplementing [$^{13}C_6$]PAA into tea leaves, the [$^{13}C_6$]CA content significantly increased compared
342 with that automatically derived from the [$^{13}C_6$]PAA standard (Figure 6B). This suggested that
343 PAA was transformed into CA in tea leaves. In-depth studies on the enzyme(s) participating in
344 the pathway are needed to obtain further evidence of the pathway from PAA to CA in tea leaves.
345 The research on the key enzymes involved in the pathway from PAA to CA is of significance.
346 However, there are two huge challenges in investigating the unknown key enzymes involved in
347 this pathway. At present, there is no available reference information on this pathway in other
348 organisms, even microorganism. Therefore, it was impossible to obtain the key genes by blasting
349 analysis. A classical purification approach is needed to identify a new enzyme. In this approach,
350 the unknown enzyme is obtained using biochemical chromatography, and the sequence
351 information of desired protein is applied to isolate the gene from the cDNA of plant.[37] However,
352 the method is time-consuming and of uncertainty, and the desired enzyme may finally not be
353 obtained. In addition, there is no stable genetic transformation system in tea plants, so it is
354 impossible to confirm the real function of new enzymes *in vivo*. The related research is of
355 interest and significance, and deserves to be carried out in the future study.
356
357 **An alternative pathway leading from L-Phe to CA also occurs in other plants**
358 To further determine whether the alternative pathway to CA was present specifically in tea
359 leaves or ubiquitous among most plants, other plants, including *R. hybrida* flowers (economic
360 crop flower), *L. esculentum* Mill fruits (economic crop fruit), and *A. thaliana* leaves (leaf model
361 plant), were investigated. Similar to tea leaves, these selected plants contained [$^{2}H_6$]CA and

362 [$^2H_7$]CA when supplied with L-[$^2H_8$]Phe (Figure 7), suggesting that the alternative pathway to
363 CA might occur in most plants, regardless of different tissues and species.
364 Phenylpropanoid compounds occur widely in the vegetable kingdom and have various
365 functions. Structural compositions, such as suberin, lignin, and other cell-wall-associated
366 phenolics, serve as physical barriers to provide plant protection against invasion from herbivores
367 and microbes. Phenylpropanoid/benzenoid volatiles and pigments (flavonoid and anthocyanin)
368 contribute to the aroma and color of fruits and flowers, and make sure pollination and seed
369 dispersal. Some defense chemicals, including condensed tannins, lignans, flavonoids,
370 isoflavonoids, and some simple phenolic compounds, possess antifeedant and antimicrobial
371 functions. Flavonoids and other phenolics act as UV protectants by absorbing UV light that can
372 damage DNA. Some other substances, such as isoflavonoids and salicylic acid (a ubiquitous
373 plant hormone), play important roles in signaling.[13,38–41] The deamination of L-Phe to give CA
374 under the action of PAL is the first step in the phenylpropanoid metabolic pathway and acts as a
375 critical regulating point between primary and secondary metabolism.[42] Therefore, CA serves as a
376 precursor of many plant-derived metabolites, and has key functions in regulating growth,
377 development, reproduction, and environmental responses of plants.[42]
378 In this study, we have provided the first chemical and biochemical evidence of CA formation
379 from L-Phe *via* PPA and PAA in tea leaves, which is leaves of economic crop, but also
380 confirmed that the alternative pathway was ubiquitous in most plants regardless of tissues,
381 including leaves of model crop, fruit of economic crop, and flower of economic crop (Figure 8).
382 Furthermore, our findings provide in-depth information on the biosynthetic pathway of CA and
383 its regulation in plant species to help guide the rational engineering of biosynthetic pathways to
384 CA and many phenolic compounds. Further detailed genetic analyses of the enzymes/genes

participating in the pathway from PAA to CA will address the biological significance of the alternative pathway to CA formation in plants.

ASSOCIATED CONTENT

Supporting information

Table S1 Primers Used in the Study.

Figure S1 Feeding Methods of Tea Leaves (A), Rose Flowers (B), Tomato Fruit (C), and Arabidopsis Leaf (D).

Figure S2 Identification of [$^{2}H_{6}$]*trans*-Cinnamic Acid (A) and [$^{13}C_{6}$]Phenyllactic Acid (B) Authentic Standard Based on NMR Analysis.

Figure S3 Amino Acid Sequence Alignment of Phenylpyruvic Acid Reductase.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CA, *trans*-cinnamic acid; PAA, phenyllactic acid; L-Phe, L-phenylalanine; PPA, phenylpyruvic acid; PPAR, phenylpyruvic acid reductase.

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Figure Caption

Figure 1 Proposed biosynthetic pathways into *trans*-cinnamic acid (CA) with isotope.

PAA, phenyllactic acid; L-Phe, L-phenylalanine; PPA, phenylpyruvic acid.

Figure 2 Determination of labeled *trans*-cinnamic acid (CA) in tea leaves fed with L-[$^2H_8$]phenylalanine (Phe).

(A) Schematic presentation of synthesis of [$^2H_6$]CA. (B) Identification of [$^2H_7$]CA and [$^2H_6$]CA standards. (C) GC–MS chromatography of [$^2H_7$]CA and [$^2H_6$]CA in tea leaves fed with L-[$^2H_8$]Phe.

Figure 3 Functional characterization of phenylpyruvic acid reductases (CsPPARs) *in vitro*.

(A) SDS-PAGE analysis of CsPPARs expressed in *Escherichia coli*. (B) Western blot analysis of CsPPARs expressed in *E. coli*. (C) Enzymatic activity analysis and substrate selectively of CsPPARs expressed in *E. coli*. N.D., not detected. Data are expressed as mean ± standard deviation (SD) (n = 3). Means with different letters are significantly different from each other ($p \leq 0.05$).

Figure 4 Functional characterization of phenylpyruvic acid reductases (CsPPARs) *in vivo*.

(A) Enzymatic activity analysis of CsPPARs transiently overexpressed in *Nicotiana benthamiana* leaves. Vector, empty vector GV3101 overexpressed in *N. benthamiana*; CsPPAR1, vector GV3101/CsPPAR1 overexpressed in *N. benthamiana*; CsPPAR2, vector GV3101/CsPPAR2 overexpressed in *N. benthamiana*. FW, fresh weight. Data are expressed as

mean ± standard deviation (SD) (n = 6). Means with different letters are significantly different from each other ($p \leq 0.05$). (B) Characteristic ions of product from phenyllactic acid (PAA) derivatized by MSTFA. (C) GC–MS chromatography of PAA in *N. benthamiana* leaves after CsPPARs overexpression.

Figure 5 Subcellular localization analyses of phenylpyruvic acid reductases (CsPPARs).

(A) Subcellular localization analyses of CsPPARs-GFP. The CsPPARs-GFP fusion proteins were transiently expressed in *Nicotiana benthamiana* leaves. Green fluorescence indicated CsPPARs-GFP fusion protein. Red showed the chloroplast auto-fluorescence, respectively. Merged panel shows overlay of gren and red fluorescence images. (B) Subcellular localization analyses of CsPPARs-YFP. The CsPPARs-YFP fusion proteins were transiently expressed in *Arabidopsis* protoplasts. Yellow fluorescence indicated CsPPARs-YFP fusion protein. Red showed the chloroplast auto-fluorescence, respectively. Merged panel shows overlay of yellow and red fluorescence images.

Figure 6 Identification of the biosynthetic pathway into *trans*-cinnamic acid (CA) from phenyllactic acid (PAA) in tea leaves.

(A) Schematic presentation of synthesis of [$^{13}C_6$]PAA. L-Phe, L-phenylalanine. (B) Change in content of [$^{13}C_6$]CA in tea leaves fed with [$^{13}C_6$]PAA. Control, sum of [$^{13}C_6$]CA content from tea leaves and [$^{13}C_6$]PAA standard; [$^{13}C_6$]PAA Treatment, [$^{13}C_6$]CA content from tea leaves. Data are expressed as mean ± standard deviation (SD) (n = 4). $^{**}p \leq 0.01$, comparison between control and treatment. FW, fresh weight.

Figure 7 GC–MS chromatography of [$^2H_7$]CA and [$^2H_6$]CA in plants fed with L-[$^2H_8$]phenylalanine (Phe).

R. hybrida, *Rosa hybrida*; *L. esculentum* Mill, *Lycopersivon esculentum* Mill; *A. thaliana*, *Arabidopsis thaliana*.

Figure 8 An alternative pathway for the formation of *trans*-cinnamic acid (CA) derived from L-phenylalanine (Phe) in plants.

AAAT, aromatic amino acid aminotransferase; PAA, phenyllactic acid; PAL, phenylalanine ammonia-lyase; PPA, phenylpyruvic acid; PPAR, phenylpyruvic acid reductases.

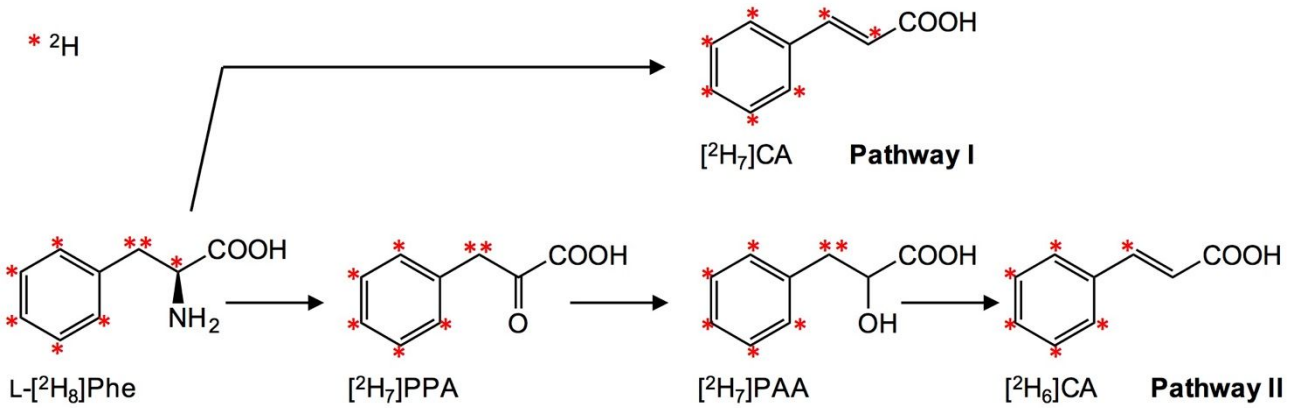

* 2H
COOH
[2H7]CA
Pathway I
COOH
NH2
L-[2H8]Phe
COOH
O
[2H7]PPA
COOH
OH
[2H7]PAA
COOH
[2H6]CA
Pathway II

Figure 1

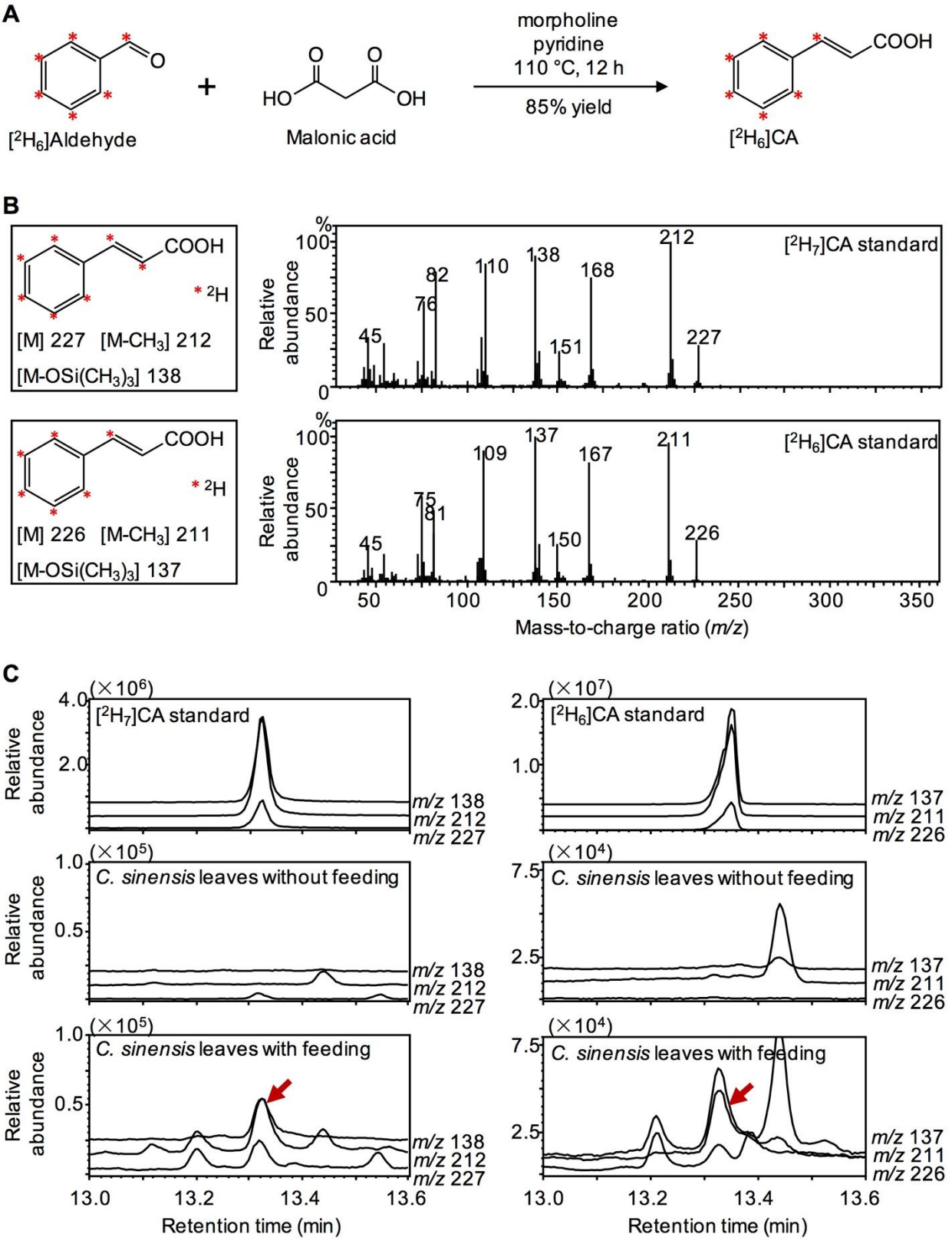

A
morpholine
pyridine
110 °C, 12 h
85% yield
[2H6]Aldehyde
Malonic acid
[2H6]CA
B
COOH
*2H
[M] 227 [M-CH3] 212
[M-OSi(CH3)3] 138
[2H7]CA standard
Relative abundance
Mass-to-charge ratio (m/z)
[M] 226 [M-CH3] 211
[M-OSi(CH3)3] 137
[2H6]CA standard
C
[2H7]CA standard
[2H6]CA standard
C. sinensis leaves without feeding
C. sinensis leaves with feeding
m/z 138
m/z 212
m/z 227
m/z 137
m/z 211
m/z 226
Retention time (min)

Figure 2

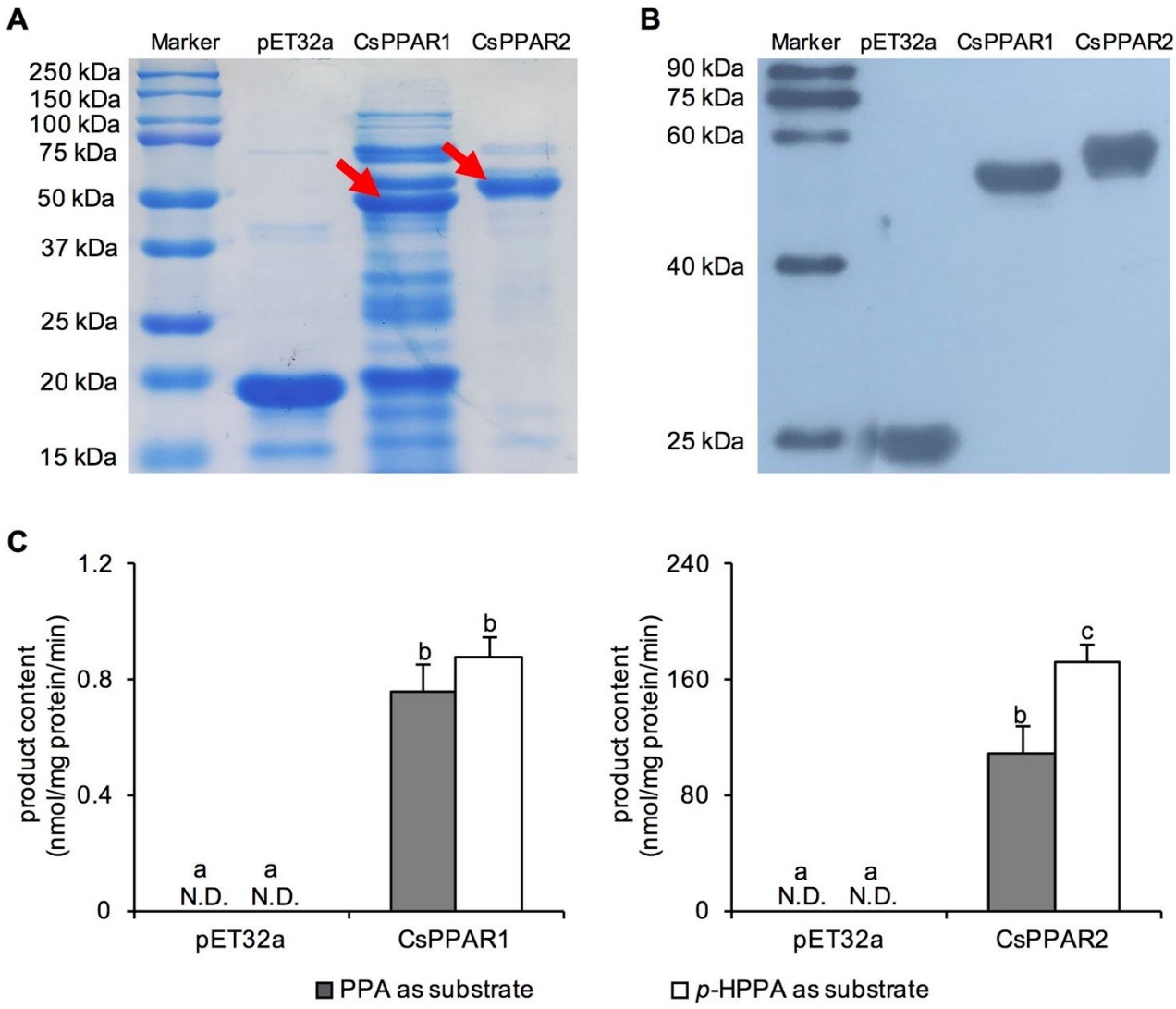

A
Marker
pET32a
CsPPAR1
CsPPAR2
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
B
Marker
pET32a
CsPPAR1
CsPPAR2
90 kDa
75 kDa
60 kDa
40 kDa
25 kDa
C
product content (nmol/mg protein/min)
1.2
0.8
0.4
0
a N.D.
a N.D.
b
b
pET32a
CsPPAR1
PPA as substrate
240
160
80
0
a N.D.
a N.D.
b
c
pET32a
CsPPAR2
p-HPPA as substrate

Figure 3

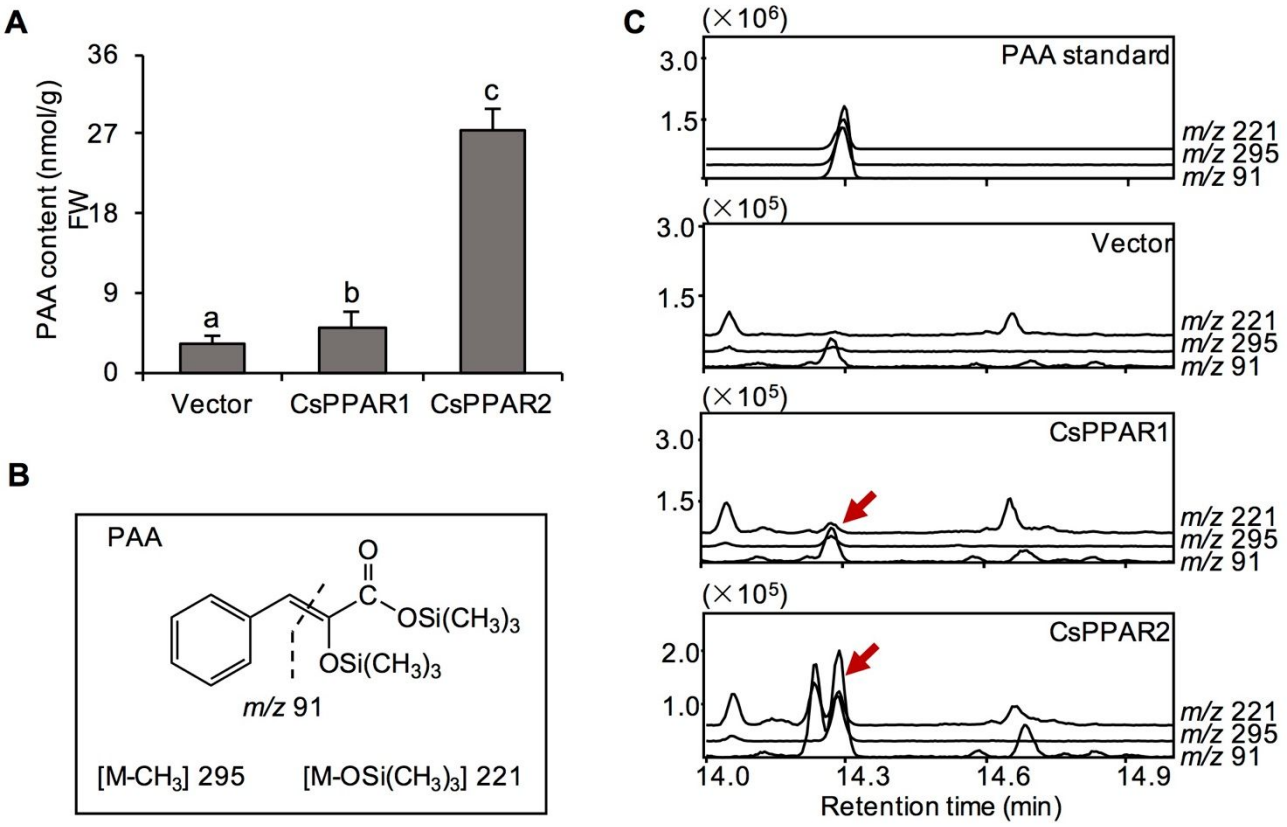

A
PAA content (nmol/g) FW
36
27
18
9
0
a
b
c
Vector
CsPPAR1
CsPPAR2
B
PAA
O
C
OSi(CH3)3
OSi(CH3)3
m/z 91
[M-CH3] 295
[M-OSi(CH3)3] 221
C
(×10^6)
PAA standard
3.0
1.5
m/z 221
m/z 295
m/z 91
(×10^5)
Vector
3.0
1.5
m/z 221
m/z 295
m/z 91
(×10^5)
CsPPAR1
3.0
1.5
m/z 221
m/z 295
m/z 91
(×10^5)
CsPPAR2
2.0
1.0
m/z 221
m/z 295
m/z 91
14.0
14.3
14.6
14.9
Retention time (min)

Figure 4

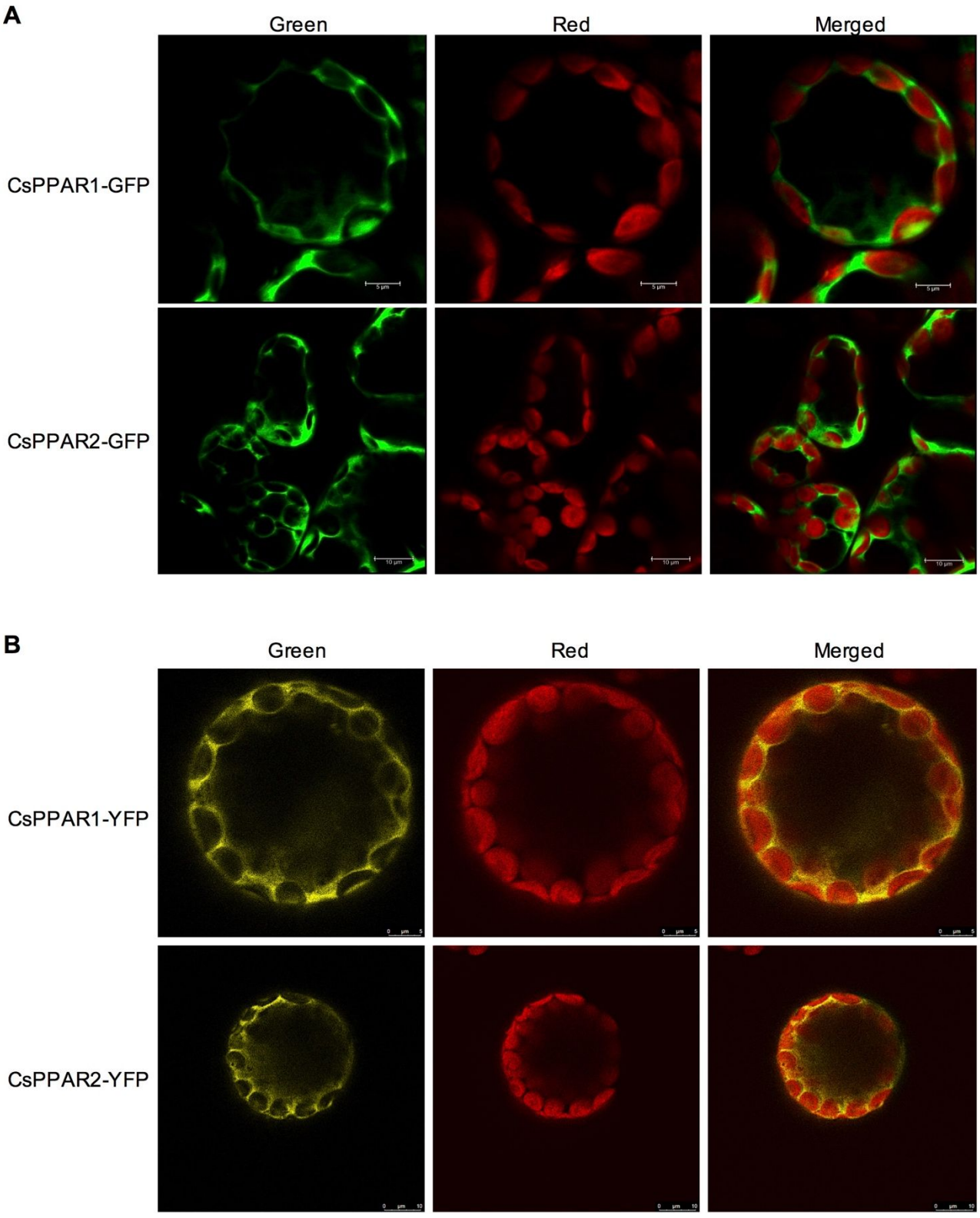

A
Green
Red
Merged
CsPPAR1-GFP
CsPPAR2-GFP
B
Green
Red
Merged
CsPPAR1-YFP
CsPPAR2-YFP

Figure 5

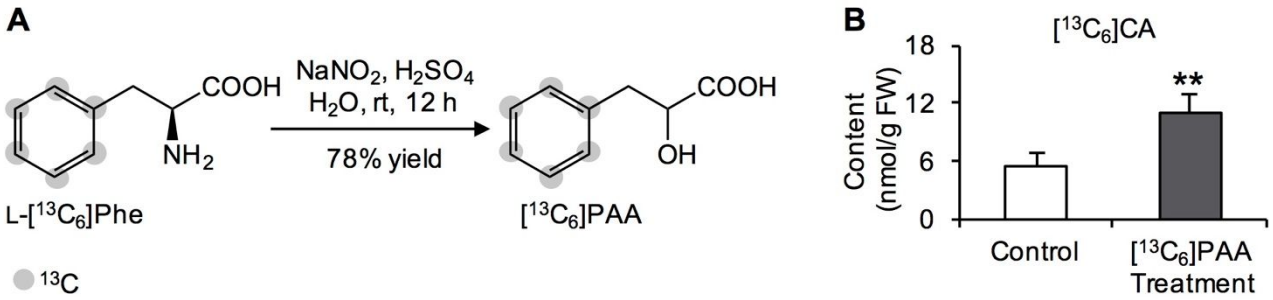

A
COOH
NH2
NaNO2, H2SO4
H2O, rt, 12 h
78% yield
COOH
OH
L-[13C6]Phe
[13C6]PAA
13C
B
[13C6]CA
Content (nmol/g FW)
18
12
6
0
**
Control
[13C6]PAA Treatment

Figure 6

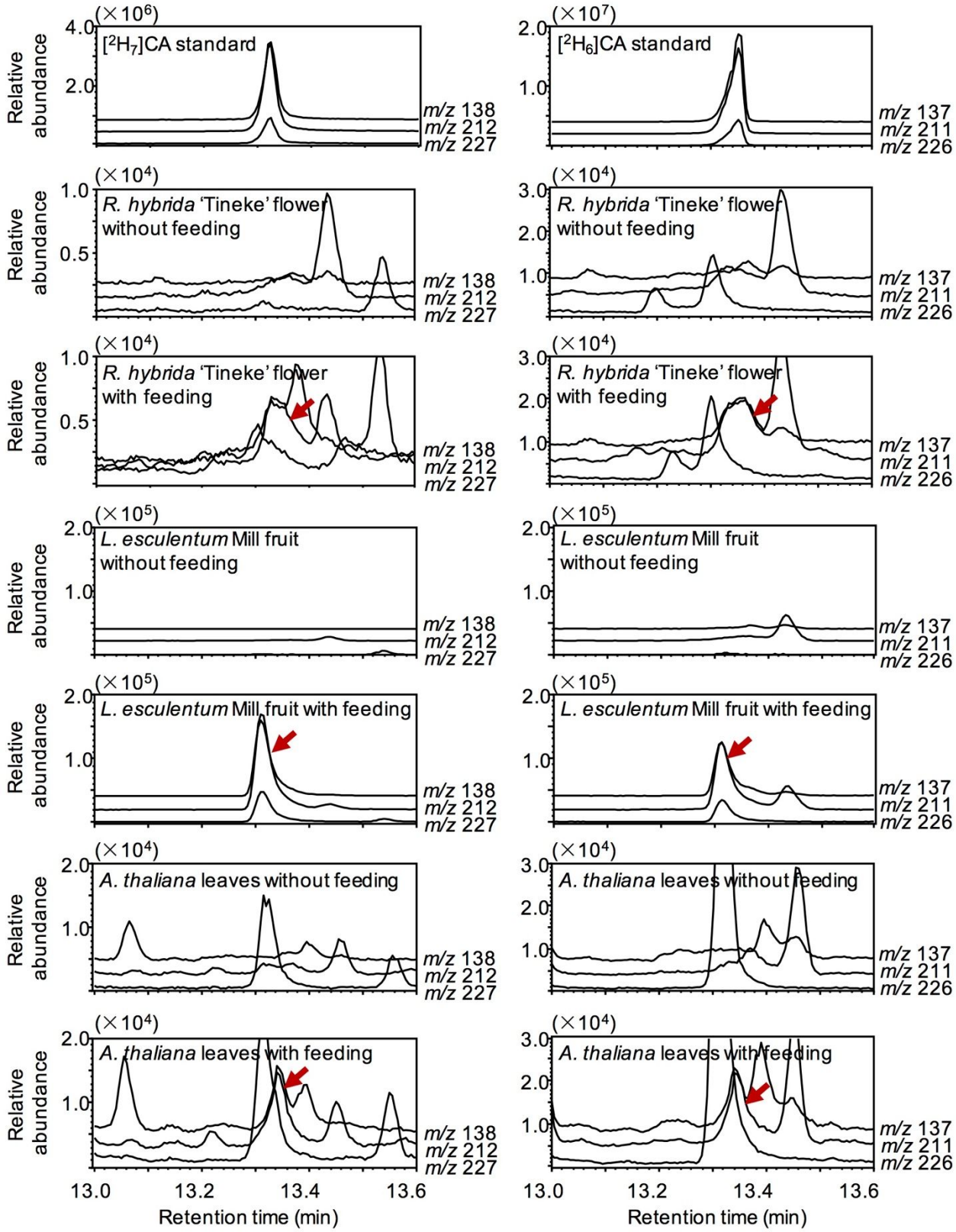

(×10^6)
[2H7]CA standard
(×10^7)
[2H6]CA standard
R. hybrida 'Tineke' flower without feeding
R. hybrida 'Tineke' flower with feeding
L. esculentum Mill fruit without feeding
L. esculentum Mill fruit with feeding
A. thaliana leaves without feeding
A. thaliana leaves with feeding
Relative abundance
m/z 138
m/z 212
m/z 227
m/z 137
m/z 211
m/z 226
13.0
13.2
13.4
13.6
Retention time (min)

Figure 7

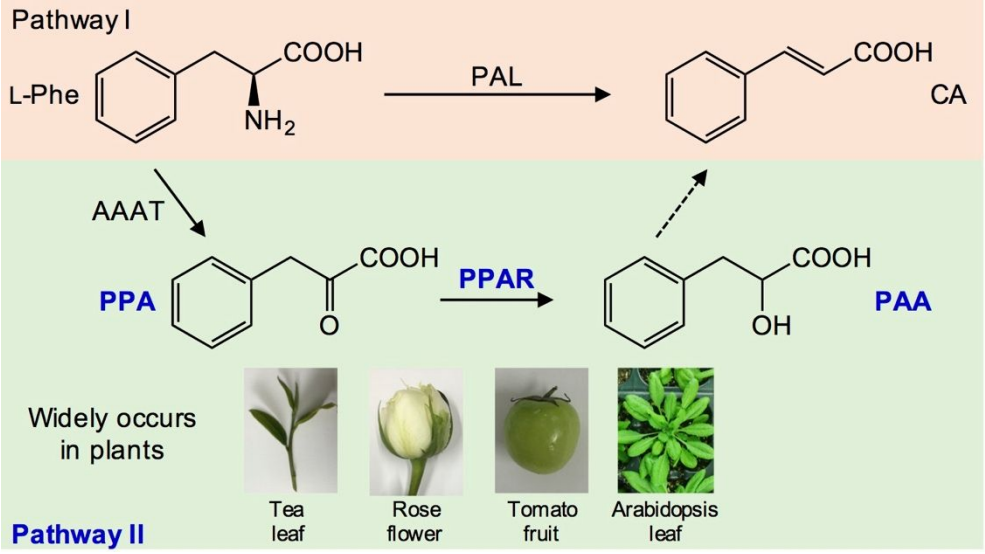
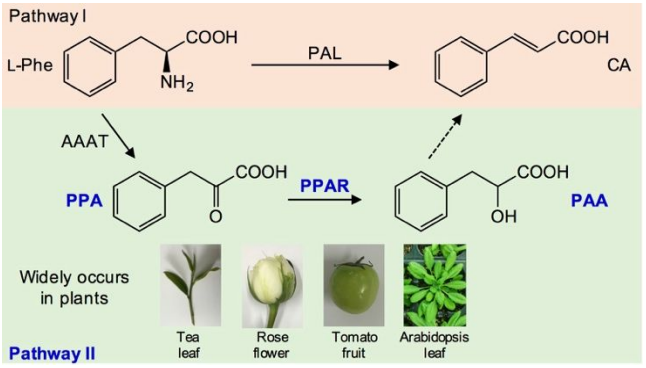

Pathway I
L-Phe
COOH
NH2
PAL
COOH
CA
AAAT
PPA
COOH
O
PPAR
COOH
OH
PAA
Widely occurs in plants
Tea leaf
Rose flower
Tomato fruit
Arabidopsis leaf
Pathway II

Figure 8

TOC


Pathway I
L-Phe
COOH
NH2
PAL
COOH
CA
AAAT
PPA
COOH
O
PPAR
COOH
OH
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Widely occurs in plants
Tea leaf
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