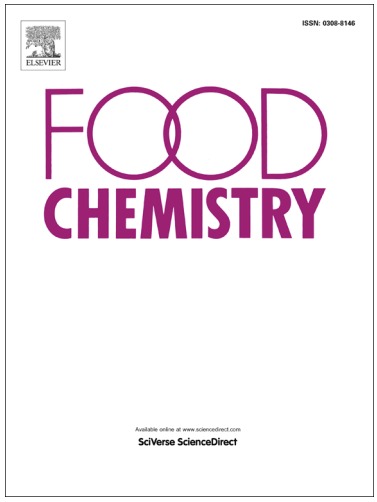

An alternative pathway for the formation of aromatic aroma compounds derived from L-phenylalanine *via* phenylpyruvic acid in tea (*Camellia sinensis* (L.) O. Kuntze) leaves

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Running title: Aromatic aroma compounds in tea

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Abstract

Aromatic aroma compounds contribute to flavor of tea (*Camellia sinensis* (L.) O. Kuntze) and they are mostly derived from L-phenylalanine *via trans*-cinnamic acid or directly from L-phenylalanine. The objective of this study was to investigate whether an alternative pathway derived from L-phenylalanine *via* phenylpyruvic acid is involved in formation of aroma compounds in tea. Enzyme reaction with phenylpyruvic acid showed that benzaldehyde, benzyl alcohol, and methyl benzoate were derived from phenylpyruvic acid in tea leaves. Feeding experiments using [$^{2}H_{8}$]L-phenylalanine indicated that phenylpyruvic acid was derived from L-phenylalanine in a reaction catalyzed by aromatic amino acid aminotransferases (AAATs). CsAAAT1 showed higher catalytic efficiency towards L-phenylalanine ($p \leq 0.001$) while CsAAAT2 showed higher catalytic efficiency towards L-tyrosine ($p \leq 0.001$). Both CsAAATs were localized in the cytoplasm of leaf cells. In conclusion, an alternative pathway for the formation of aromatic aroma compounds derived from L-phenylalanine *via* phenylpyruvic acid occurred in tea leaves.

Keywords: Aroma; *Camellia sinensis*; Phenylalanine; Phenylpyruvic acid; Tea; Volatile

Abbreviations:

AAATs, aromatic amino acid aminotransferases; AIP, 2-aminoindan-2-phosphonic acid; DTT, dithiothreitol; GC-MS, gas chromatography-mass spectrometry; MSTFA, *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide; PLP, pyridoxal phosphate; PVPP, polyvinylpolypyrrolidone; TMEMD, *N, N, N', N*'-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; SPME, solid-phase microextraction; UPLC-QTOF-MS, ultra performance liquidchromatography

coupled with quadrupole time-of-flight mass spectrometry; VPBs, volatile phenylpropanoids/ benzenoids.

1. Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) volatile compounds contribute to the aroma property of tea flavour, and determine the final quality of the tea product. Besides non-volatile compounds including catechins, amino acids, and caffeine, tea volatile compounds are also representative specialized metabolites in tea leaves (Wan, & Xia, 2015). Our current knowledge of the biosynthesis of tea volatiles has been obtained substantially from studies on other plant species. Similar to volatiles in other plants, tea volatiles can be classified into four major classes according to their metabolic origin: volatile terpenoids, volatile phenylpropanoids/benzenoids (VPBs, also known as aromatic aroma compounds), volatile fatty acid derivatives, and volatiles derived from carotenoids (Yang, Baldermann, & Watanabe, 2013). Some plant volatile formation pathways are shared among different plant species, but some differ among plant species because of the complex networks of plant volatile biosynthesis. Therefore, direct evidence of volatiles biosynthesis in tea leaves is required. As a genetic transformation system has not yet been established for tea, it is difficult to study the biosynthetic pathways of specialized metabolites in tea leaves in detail. Consequently, few enzymes related to tea aroma have been functionally characterized. β-Glycosidases including β-primeverosidases and β-glucosidases, and glycosyl transferases, which are involved in the transformation between glycoside-bound volatiles and free volatiles in tea leaves, have been intensively studied and functionally characterized (Mizutani et al., 2002; Zhou et al., 2014; Ohgami et al., 2015). Recently, several enzymes and genes involved in the final steps of the biosynthesis of tea aroma compounds including

(*S*)-linalool, (*E*)-nerolidol, indole, and methyl salicylate, have been isolated, identified, and functionally characterized (Fu et al., 2015; Zeng et al., 2016; Mei et al., 2017; Zhou et al., 2017; Liu et al., 2018). In contrast, little attention has been paid to upstream pathways in the formation of tea aroma compounds, especially VPBs.

The VPBs are the second most ubiquitous class of plant volatiles and have important ecological functions and potential economic applications, for example, in sedation, improvement of memory, and improvement of food storage and flavor (Pichersky, Noel, & Dudareva, 2006; Schwab, Davidovich-Rikanati, & Lewinsohn, 2008). Most volatiles in this class contain an aromatic ring and originate from shikimate *via* L-phenylalanine. The well-known upstream pathway of VPBs formation is the deamination of L-phenylalanine to *trans*-cinnamic acid, catalyzed by L-phenylalanine ammonia lyase (Pichersky, Noel, & Dudareva, 2006; Schwab, Davidovich-Rikanati, & Lewinsohn, 2008) (Figure 1A). However, the two VPBs phenylacetaldehyde and 2-phenylethanol are not produced from *trans*-cinnamic acid and are directly derived from L-phenylalanine (Tieman et al., 2006) (Figure 1A). Recent studies have indicated that phenylpyruvic acid may also be involved in the formation of VPBs, as validated in melon, rose flowers, and tea flowers (Gonda et al., 2010; Dong et al., 2012; Hirata et al., 2012). However, it is still unknown whether phenylpyruvic acid is involved in the formation of VPBs in tea leaves. To answer this question, we supplied different substrates to enzyme extracts of tea leaves, and found that benzaldehyde, benzyl alcohol, and methyl benzoate were derived from phenylpyruvic acid in tea leaves. Then, stable isotope-labeled [$^{2}H_{8}$]L-phenylalanine was supplied to tea leaves to investigate the phenylpyruvic acid pathway. Finally, the gene involved in phenylpyruvic acid formation was isolated, cloned, sequenced, and functionally characterized. The aim of this study was to discover the biosynthesis of phenylpyruvic acid in tea leaves and

the role of phenylpyruvic acid in the formation of tea aroma, especially VPBs.

2. Materials and methods

2.1. Chemicals and regents

2-Aminoindan-2-phosphonic acid (AIP) was purchased from Absin Bioscience Inc., Shanghai, China. Benzaldehyde, benzyl alcohol, acetophenone, 2-phenylethanol, methyl salicylate, *trans*-cinnamic acid, and ethyl decanoate were purchased from Wako Pure Chemical Industries Ltd., Japan. EasySee Western Marker (25-90 kDa) was purchased from TransGen Biotech, Beijing. ECL kit, Precision Plus Protein standard (10-250 kDa), 2 × SYBR Green Universal PCR Mastermix, *N, N, N', N*'-tetramethylethylenediamine (TMEMD), 30% acrylamide/bis solution, and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Laboratories, Hercules, CA, USA. [α,β,β,2,3,4,5,6-$^{2}H_{8}$]L-Phenylalanine was purchased from Cambridge Isotope Laboratories Inc., Cambridge, MA, USA. Methyl benzoate was purchased from Shanghai Macklin Biochemical Co., Ltd., China. Ni-NTA resin was purchased from Qiagen Inc., USA. *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from Regis Chemical Co., Morton Grove, Illinois, USA. PD-10 desalting column was purchased from GE Healthcare Life Sciences, Chandler, Arizona, USA. Phenylpyruvic acid and polyvinylpolypyrrolidone (PVPP) were purchased from Sigma-Aldrich Company Ltd., St. Louis, MO, USA. Quick RNA isolation kit was purchased from Huayueyang Biotechnology Co., Ltd., China.

2.2. Plant materials

Tea plants of *C. sinensis* cv. Jinxuan were widely cultivated in South China and the experiment

materials were acquired from Yingde Tea Experimental Station of Tea Research Institute, Guangdong Academy of Agricultural Sciences (23°N, 113°E, Yingde, China).

2.3. Extraction of enzymes from tea leaves and analyses of enzymatic products

The L-phenylalanine transaminase assay was conducted according to a literature method (Gonda et al., 2010) with slight modifications. The detailed enzyme extraction method is shown in the 'Supplementary Information'. A reaction mixture (400 μL) containing 5 mM [$^2H_8$]L-phenylalanine and crude enzyme solution (350 μL) was incubated at 30 °C for 60 min, then acidified with 1 M hydrochloric acid (80 μL), and then incubated for another 30 min at 30 °C. The samples were extracted with ethyl acetate (800 μL), and then centrifuged at 2,500 ×*g* to separate the phases. The organic phase was collected, blown dry under a stream of nitrogen gas and derivatized with MSTFA (80 μL) at 37 °C for 60 min. The MSTFA-derivated sample (1 μL) was analyzed by gas chromatography–mass spectrometry (GC-MS) (The GC-MS analysis condition is described in the 'Supplementary Information').

The phenylpyruvic acid and *trans*-cinnamic acid conversion assays were conducted as described previously (Hirata et al., 2012) with slight modifications. The detailed enzyme extraction method is shown in the 'Supplementary Information'. A reaction mixture (400 μL) containing 5 mM phenylpyruvic acid or *trans*-cinnamic acid (dissolved in 5% (v/v) methanol), and crude enzyme solution (350 μL) was incubated at 30 °C for 60 min, and then placed on ice for 10 min to stop the reaction. To determine internal volatiles, the mixture was extracted with an equal volume hexane/ethyl acetate (1:1) containing ethyl decanoate (5 nmol). Headspace volatiles were collected using a solid-phase microextraction (SPME) fiber (2 cm-50/30 μm DVB/Carboxen^{TM}/ PDMS Stable Flex^{TM}, Supelco Inc., Bellefonte, PA, USA). The SPME fiber

or 1 μL of the extract was subjected to GC-MS analysis (The GC-MS analysis condition is described in the 'Supplementary Information').

2.4. Supplementation of [$^2H_8$]L-phenylalanine or cosupplementation of [$^2H_8$]L-phenylalanine and AIP to tea leaves and identification of deuterium labeled products

Individual tea sample (one bud and two or three leaves) was injected with 4 μmol [$^2H_8$]L-phenylalanine and 0.4 μmol AIP. Samples treated only with [$^2H_8$]L-phenylalanine served as the control group. All samples were kept in an incubator under a 16-h light/8-h dark photoperiod at 70% humidity and 25 ℃ for 3 days, and then immediately frozen in liquid nitrogen and stored at -80 ℃ until further analyses. Four independent replicates were performed.

The labeled phenylpyruvic acid product was identified using a literature method (Dong et al., 2012) with slight modifications. Tea samples (0.8 g, finely powdered) were extracted with cold methanol (4 mL) by vortexing for 2 min followed by ultrasonic extraction in ice cold water for 10 min. Each extract was mixed with cold chloroform (4 mL) and cold water (1.6 mL), and the phases were allowed to separate. The upper layer was blown dry under a stream of nitrogen gas to remove the organic solvent. The water extract was mixed with 0.25 M hydrochloric acid (50 μL) and extracted twice with ethyl acetate (1.0 mL). The ethyl acetate extract was blown dry under a stream of nitrogen gas and derivatized with MSTFA (80 μL) at 37 ℃ for 60 min. The MSTFA-derivated samples were then analyzed by GC-MS (The GC-MS analsis condition is described in the 'Supplementary Information').

To analyze the labeled volatiles, tea samples (200 mg, finely powdered) were extracted by 1 mL dichloromethane containing of ethyl decanoate (5 nmol) as an internal standard in a shaker at room temperature for 6 hours. The extraction solution was dried over anhydrous sodium sulfate

and then a 1-μL aliquot was subjected to GC-MS analysis (The GC-MS analysis condition is described in the 'Supplementary Information').

2.5. Phylogenetic analyses of CsAAATs and AAATs from various plant species

The protein sequence of *Cucumis melo* L. *CmArAT1*, a phenylalanine transaminase (GenBank: FJ896816.1), was used for a BlastN searching in the Transcriptome Shotgun Assembly (TSA) database of *C. sinensis*. Two aromatic amino acid transaminases members, *CsAAAT1* (accession no: MH544095) and *CsAAAT2* (accession no: MH544096), were highly familiar and selected from the TSA database online. Phylogenetic analysis was conducted and viewed using MEGA 7.0 software on the basis of the neighbor–joining algorithms with a bootstrap test of 1000 replicates.

2.6. CsAAATs recombinant expression and western blot analyses

The full length open reading frames (ORFs) of *CsAAAT1* and *CsAAAT2* were amplified by PCR with the primers listed in Table S1 (Supplementary Information). For CsAAATs recombinant expression, the detailed method is described in the 'Supplementary Information'.

Western blot analyses were performed according to a literature method (Mahmood, & Yang, 2012). Protein samples were electrophoresed on a SDS–polyacrylamide gel containing a separation part (2700 μL of ddH_2O, 2400 μL of 30% acrylamide/bis (acrylamide), 1800 μL of Tris-HCl (pH 8.8), 70 μL of 10% SDS, 70 μL of 10% ammonium persulfate, and 3 μL of TMEMD) and a concentration part (1400 μL of ddH_2O, 340 μL of 30% acrylamide/bis (acrylamide), 250 μL of Tris-HCl (pH 6.8), 20 μL of 10% SDS, 20 μL of 10% ammonium persulfate, and 2 μL of TMEMD). Precision Plus Protein standard (10-250 kDa) and EasySee

Western Marker (25-90 kDa) were used as molecular weight marker. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 and destained with dye-destaining solution. Following SDS-PAGE analysis, the proteins were transferred from a gel onto a membrane (polyvinylidine fluoride (PVDF), 0.2 μm pore size; NuPAGE®) and then labeled with a His-Tag mouse monoclonal antibody (Signalway antibody, Pearland, TX, USA) at a dilution of 1:5000, continued with a HRP-conjugated goat anti-mouse IgG (Signalway antibody, Pearland, TX, USA) diluted at 1:10000. Immunoreactive spots were detected using the ECL kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

2.7. CsAAATs recombinant enzyme assay and substrate selectivity

CsAAATs recombinant enzyme assay was conducted according to a literature method (Gonda et al., 2010) with slight modifications.The reaction mixture (1 mL) contained purified protein (25 μg of protein), and 50 mM BIS-TRIS propane buffer (pH 8.5) containing 10 mM α-ketoglutarate, 10 mM L-phenylalanine or L-tyrosine, 1 mM dithiothreitol (DTT), 225 μM pyridoxal phosphate (PLP), and 10% (w/v) D-sorbitol. The reactions were incubated at 30 °C for 2 h. The products (phenylpyruvic acid derived from L-phenylalanine and 4-hydroxyphenylpyruvic acid derived from L-tyrosine) were extracted twice with ethyl acetate (2 × 1 mL). The ethyl acetate extract was blown dry under a stream of nitrogen gas, and then re-dissolved in acetonitrile (200 μL). After filtering through a 0.22-μm membrane, the ten-fold diluted extract was analyzed by an ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS; ACQUITY UPLC I-Class/Xevo® G2-XS QTOF, Waters Corp., Milford, MA, USA). The UPLC-QTOF-MS analysis conditions are described in the 'Supplementary Information'. The compounds were identified from their retention time and MS data

(phenylpyruvic acid, t_R = 5.13 min, *m/z* 163.0395 $[M-H]^-$; 4-hydroxyphenylpyruvic acid, t_R = 3.12 min, *m/z* 179.0344 $[M-H]^-$).

2.8. Functional identification and subcellular localization of CsAAATs in vivo

2.8.1. Agrobacterium-mediated transient expression of CsAAATs in Nicotiana benthamiana

The primers used to amplify the sequences to express the GFP-fusion protein are listed in Table S1 (Supplementary Information). The reorganization vectors were transformed into *Agrobacterium* GV3101 by electroporation according to the manufacturer's instructions. The detailed method is described in the 'Supplementary Information'.

2.8.2. Analyses of CsAAATs activities in overexpressed Nicotiana benthamiana

To analyze *CsAAATs* activities in overexpressed *Nicotiana benthamiana*, tobacco leaves (0.8 g) of *Nicotiana benthamiana* (finely powdered) were used for phenylpyruvic acid extraction and detection using the method described above.

2.8.3. Subcellular localization of CsAAATs

Tobacco leaves were cut into 1-2 cm^2 pieces, and confocal microscope images were taken using a Zeiss LSM 510 META confocal laser microscope (Carl Zeiss, Jena, Germany) with a 40× water objective under an excitation wavelength of (488 nm) for GFP fluorescence observation.

2.9. Transcript expression analyses of CsAAATs in different tissues

Total RNA was extracted from roots, stems, young leaves, mature leaves, and flowers of *C.*

sinensis, respectively, using a Plant Total RNA Kit (Huayueyang Biotechnology CO., Ltd., China). The first-strand cDNA was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Industry CO., Ltd., Japan). Gene transcript expression was quantified using quantitative real-time PCR (qRT-PCR). The reaction (20 μL) was consisted of iTaq Universal SYBR Green Supermix (10 μL, 2×, Bio-Rad, Hercules, CA, USA), the specific forward and reverse primers (0.2 μM each), and the 20-fold diluted template (2 μL). The encoding elongation factor 1 (*EF1*) was used as an internal reference gene (Hao et al., 2014). The *CsEF1*, *CsAAAT1* and *CsAAAT2* gene specific primers of qRT-PCR are listed in Table S1 (Supplementary Information). The qRT-PCR was conducted on a Roche LightCycle 480 system (Roche Applied Science, Mannheim, Germany) under the following conditions: One cycle of 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A melt curve was obtained for each sample at the end of each run to ensure the purity of the amplified products. The $2^{-\Delta\Delta C}{}_{T}$ method was used to measure the relative expression level (Livak et al., 2001). Changes in mRNA levels of *CsAAAT1* and *CsAAAT2* were normalized to that of *CsEF1*. Each tissue point represents an average of three independent biological samples.

2.10. Statistical analysis

Statistical analysis was performed using SPSS Statistics 23.0 software. The statistical significances of the differences between two treatments were calculated using a two-tailed Student's *t*-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). One-way ANOVA followed by Duncan's multiple comparison tests was used to determine the differences among three or more than three groups. A probability level of 5% ($p \leq 0.05$) was defined as significant.

3. Results and discussion

3.1. Benzaldehyde, benzyl alcohol, and methyl benzoate were derived from phenylpyruvic acid in tea leaves

Based on previous reports (Dudareva, Negre, Nagegowda, & Orlova, 2006; Koeduka et al., 2006), the upstream pathway of VPBs formation is mainly *via trans*-cinnamic acid. However, recent studies (Orlova et al., 2006; Yoo et al., 2013; de la Torre, El-Azaz, Ávila, & Cánovas, 2014) have suggested that there might be an alternative pathway from another precursor, possibly phenylpyruvic acid (Figure 1A). To investigate whether phenylpyruvic acid is involved in the formation of VPB compounds in tea leaves, we prepared a crude enzyme extract from tea leaves and monitored the reactions using *trans*-cinnamic acid and phenylpyruvic acid as substrates. Then, we detected the changes in the contents of the related aroma compounds. The contents of 2-phenylethanol, acetophenone, and methyl salicylate increased when using phenylpyruvic acid as a substrate, but no statistically significant differences were detected between the CK and treatment group (Figure S1). However, benzaldehyde, benzyl alcohol, and methyl benzoate significantly accumulated in the reaction using phenylpyruvic acid as the substrate (Figure 1B), whereas the contents of these three compounds were almost unchanged when using *trans*-cinnamic acid as the substrate (Figure 1C). These results provided *in vitro* evidence that three important VPBs (benzaldehyde, benzyl alcohol, and methyl benzoate) are derived from phenylpyruvic acid in tea leaves. Because phenylpyruvic acid is unstable, an isotopically labeled form of this compound is not commercially available. To further obtain *in vivo* evidence, we extracted and analyzed the aroma compounds from tea leaves after supplementation with [$^{2}H_{8}$]L-phenylalanine or co-supplementation with [$^{2}H_{8}$]L-phenylalanine

and AIP, a potent inhibitor of phenylalanine ammonia lyase. Labeled benzaldehyde was detected in tea leaves after supplementation with [$^2H_8$]L-phenylalanine (Figure S2). Howevere, there was no obvious difference in the amounts of labeled benzaldehyde and benzyl alcohol between the two treatment groups including supplementation with [$^2H_8$]L-phenylalanine and co-supplementation with [$^2H_8$]L-phenylalanine and AIP (Figure 1D), and labeled methyl benzoate was not detected. The *in vivo* test further confirmed that phenylpyruvic acid was the precursor of benzaldehyde and benzyl alcohol in tea leaves. Labeled methyl benzoate was not detected, possibly because it was beneath the limits of detection. In most plants, many important VPBs are derived from *trans*-cinnamic acid (Dudareva, Negre, Nagegowda, & Orlova, 2006). However, several studies have shown that phenylpyruvic acid is also likely to be an important precursor of VPB compounds in some plants, such as melon (*Cucumis melo*) (Gonda et al., 2010), rose flowers (*Rosa* × *hybrida* 'Yves Piaget') (Hirata et al., 2012), and tea flowers (Dong et al., 2012). Our study is the first confirmation that phenylpyruvic acid is an important precursor of VPB compounds in tea leaves.

Benzaldehyde and benzyl alcohol are two prominent scent compounds that greatly contribute to the fruity, floral smells of flowers, fruits, and plants. Benzaldehyde has a typical almond-like odor and is one of the key volatile components in all types of tea, especially green tea, oolong tea, and black tea (Yang, Baldermann, & Watanabe, 2013). Benzyl alcohol also largely contributes to the floral aroma of oolong tea and black tea (Mizutani et al., 2002). Methyl benzoate is one of essential floral compounds of many bee-pollinated flowers, for instance snapdragon flowers (Pichersky, & Gershenzon, 2002). However, the upstream pathway in the synthesis of benzaldehyde, benzyl alcohol, and methyl benzoate remains unclear. In micro-organisms, especially yeasts, phenylpyruvic acid is the precursor for benzaldehyde, benzyl alcohol, and

methyl benzoate, which contributes to the flavor of cheese and alcohol (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Etschmann, Bluemke, Sell, & Schrader, 2002). Some reports have speculated that these compounds may be derived from phenylpyruvic acid in plants (Orlova et al., 2006; Oliva et al., 2017) like in microorganisms, but there is no direct evidence for this. Our study has provided the first evidence that phenylpyruvic acid was the precursor of benzaldehyde, benzyl alcohol, and methyl benzoate in tea leaves.

3.2. Phenylpyruvic acid was derived from L-phenylalanine in tea leaves

To investigate whether phenylpyruvic acid is derived from L-phenylalanine in tea leaves, we carried out both *in vitro* and *in vivo* tests. First, we conducted the *in vitro* crude enzyme reaction using [$^2H_8$]L-phenylalanine as the substrate and analyzed the products. Labeled phenylpyruvic acid was detected by GC-MS, based on the detection of peaks of the characteristic ion (*m/z* 225, 299 and 314) compared with the retention time of authentic phenylpyruvic acid. The labeled phenylpyruvic acid content was calculated from the peak area of the characteristic ion *m/z* 299 (Figure 2A). The results revealed that L-phenylalanine was converted to phenylpyruvic acid by enzymes in tea leaves. To confirm this result, we carried out an *in vivo* experiment by feeding the stable isotope [$^2H_8$]L-phenylalanine to tea leaves. Labeled phenylpyruvic acid was also detected after supplementation of [$^2H_8$]L-phenylalanine to tea leaves (Figure 2B & 2C), consistent with the isotopic tracer results from rose flowers and tea flowers (Dong et al., 2012; Hirata et al., 2012). Since a genetic transformation system has not yet been established for tea, it is very difficult to obtain direct *in vivo* evidence of secondary metabolite synthesis. Therefore, the use of stable isotope-labeled secondary metabolites of precursors for *in vivo* tracing can provide sensitive and direct evidence of metabolic networks in plants for which no genetic

transformation system is available (Chokkathukalam, Kim, Barrett, Breitling, & Creek, 2014). The stable isotope labeling method has been successfully used to analyze tea secondary metabolites pathways in other studies (Zeng et al., 2016; Cheng et al., 2017). In our study, we found that phenylpyruvic acid was produced from L-phenylalanine in tea leaves *via* enzyme assays and stable isotope labeling experiments.

3.3. CsAAAT1 was involved in the pathway leading from L-phenylalanine to phenylpyruvic acid in tea leaves

To further explore the key genes and enzymes involved in the formation of phenylpyruvic acid from L-phenylalanine, we conducted BlastN searches to find putative *CsAAATs* sequences in *C. sinensis*. Data mining of the TSA database revealed two unique aromatic amino acid transaminases members, *CsAAAT1* and *CsAAAT2* (Figure 3). *CsAAAT1* was more closely related to *CmArAT1*, which encodes an L-phenylalanine transaminase (Figure 3). The two recombinant proteins were overexpressed in BL21 *E. coli* competent cells (Figure 4A). The *CsAAAT1* sequence was a full-length clone encoding a 421-amino-acid protein with a native molecular mass of 46.75 kDa, and the *CsAAAT2* sequence was a full-length clone encoding a 421-amino-acid protein with a native molecular mass of 46.2 kDa, as predicted by ExPASy (https://www.expasy.org/). Figure 4B shows the western blot analyses of CsAAAT1 and CsAAAT2 expressed successfully in *E. coli*. The semi-purified CsAAAT1 and CsAAAT2 were able to catalyze the L-phenylalanine transamination reaction to synthesize phenylpyruvic acid. Furthermore, CsAAAT1 showed higher L-phenylalanine transamination activity while CsAAAT2 showed higher tyrosine transamination activity (Figure 5A).

To obtain *in vivo* evidences of CsAAATs functions, CsAAAT1 and CsAAAT2 were introduced

into the *A. tumefaciens* strain GV3101 and transiently overexpressed in leaves of *N. benthamiana*. Both enzymes showed phenylpyruvic acid production activity *in vivo* (Figure 5B). We measured the transcript levels of *CsAAAT1* and *CsAAAT2* in different tea tissues by qRT-PCR, and detected higher levels of *CsAAAT1* transcripts in mature leaves, young leaves, and flowers (Figure S3A), and higher levels of CsAAAT2 transcripts in flowers (Figure S3B). In subcellular localization analyses, both CsAAAT1 and CsAAAT2 were located in the cytoplasm of leaf cells (Figure 5C).

The AAATs can be divided into three families; phenylalanine aminotransferases, tryptophan aminotransferases, and tyrosine aminotransferases (Wang, & Maeda, 2017). Several AAATs have been reported in plants (Lopukhina, Dettenberg, Weiler, & Holländer-Czytko, 2001; Stepanova et al., 2008; Gonda et al., 2010). The AAATs are PLP-dependent enzymes (Alexander, Sandmeier, Mehta, & Christen, 1994) that catalyze the transamination reaction of aromatic amino acids to their corresponding keto acids, and the products of AAATs in plants serve as key precusors of secondary metabolites, such as the hormone auxin, phenylpropanoids, and alkaloids (Facchini, 2001; Vogt, 2010). Phenylalanine aminotransferases are a subset of the tyrosine aminotransferase family. They are localized outside of plastids, and interlink phenylalanine and tyrosine metabolism. In our study, we sequenced and identified two AAATs that were localized in the cytoplasm of leaf cells. A phylogenetic analysis and substrate selectivity assays showed that CsAAAT1 was a phenylalanine aminotransferase while CsAAAT2 was a tyrosine aminotransferase. These results showed that CsAAAT1 was the key enzyme catzlyzing the conversion of L-phenylalanine into phenylpyruvic acid in tea leaves.

4. Conclusions

Our results showed that benzaldehyde, benzyl alcohol, and methyl benzoate in tea leaves were

derived from L-phenylalanine *via* phenylpyruvic acid (Figure 6). Two unique *CsAAATs* were sequenced and functionally characterized. Both *in vitro* and *in vivo* analyses verified that the two CsAAATs had L-phenylalanine transaminase functions. Substrate selectivity assays showed that CsAAAT1 was a L-phenylalanine aminotransferase and CsAAAT2 was a L-tyrosine aminotransferase. Subcellular localization analyses of CsAAATs showed that both were located in the cytoplasm of leaf cells. Our study clarifies the pathway of volatile phenylpropanoids/benzenoids synthesis in plants, and is the first direct evidence that benzaldehyde, benzyl alcohol, and methyl benzoate are derived from phenylpyruvic acid in tea leaves.

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Competing financial interests

The authors declare no competing financial interests.

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Figure Legends

Figure 1. Identifications of precursor of some volatile phenylpropanoids and benzenoids in tea leaves.

(A) Potential pathways of volatile phenylpropanoids and benzenoids synthesis from L-phenylalanine in plants. (B and C) Changes in amounts of benzaldehyde, benzyl alcohol, and methyl benzoate using phenylpyruvic acid (PPA) or *trans*-cinnamic acid (CA) as substrate, respectively. CK1, sum of contents volatiles produced from reaction without crude enzyme and reaction without phenylpyruvic acid. CK2, sum of contents volatiles produced from reaction without crude enzyme and reaction without *trans*-cinnamic acid. [a], contents of the products extracted from 400 μL enzyme solution after 60 min enzyme reaction; [b], contents of the products absorbed on SPME for 60 min collection after 60 min enzyme reaction. (D) Amounts of labeled benzaldehyde and benzyl alcohol in tea leaves after supplementation with [$^2H_8$]L-phenylalanine ([$^2H_8$]Phe) and co-supplement of [$^2H_8$]Phe and 2-aminoindan-2-phosphonic acid (AIP). Data are expressed as means ± S.D. (n = 3). *, $p \leq 0.05$) and **, $p \leq 0.01$.

Figure 2. Transformation from L-phenylalanine into phenylpyruvic acid *in vitro* and *in vivo* in tea leaves.

(A) Production of labeled phenylpyruvic acid (PPA) contents after crude enzyme reaction. CK, crude enzyme reaction without [$^2H_8$]L-phenylalanine; Treatment, crude enzyme reaction using [$^2H_8$]L-phenylalanine as substrate. N.D., not detected. Data are expressed as means ± S.D. (n = 3). ***, $p \leq 0.001$. (B) Characteristic ions of products from phenylpyruvic acid/labeled phenylpyruvic acid derivatized by MSTFA. (C) GC-MS chromatography of phenylpyruvic

acid/labeled phenylpyruvic acid.

Figure 3. Phylogenetic tree of CsAAATs and AAATs from other plants.

Multiple alignments were performed with MEGA 7.0. *At, Arabidopsis thaliana*; *Cm, Cucumis melo* (melon)*; Cs, Camellia sinensis; Os, Oryza sativa; Pf, Perilla frutescens; Ry, Rosa* 'Yves Piaget'; *Sm, Salvia miltiorrhiza; Ss, Solenostemon scutellarioides; Sp, Solanum pennellii* (tomato); *Zm, Zea mays.* TyrATs, tyrosine aminotransferases; PheATs, phenylalanine aminotransferases; AlaATs, alanine aminotransferases; TrpATs, tryptophan aminotransferases. Accession numbers are as follows: AtTAT1, NP_200208.1; AtTAT2, NP_198465.3; AtTrpAT, AEE35079.1; CmArAT1, NP_001284465.1; CmBCAT, ADC45390.1; CsAAAT1, MH544095; CsAAAT2, MH544096; OsAlaAT, BAA77261.1; PfTAT, ADO17550.1; RyAAAT3, BAF64843.1; SmTAT, ABC60050.1; SpTAT1, ADZ24702.1; SsTAT, CAD30341.1; ZmAlaAT, AAC62456.1; ZmTrpAT, AMD16119.1.

Figure 4. Identification of CsAAAT1 and CsAAAT2 recombinant proteins expressed in *Escherichia coli*.

(A) SDS-PAGE analysis of *E. coli*-expressed CsAAAT1 and CsAAAT2. Arrows indicate target proteins. (B) Western blot analyses of CsAAAT1 and CsAAAT2 expressed in *E. coli.*

Figure 5. Functional identification and subcellular localization of CsAAAT1 and CsAAAT2.

(A) Enzyme activities of CsAAAT1 and CsAAAT2 expressed in *Escherichia coli*. Phe as sub., enzyme reaction using L-phenylalanine as substrate; Tyr as sub., enzyme reaction using L-tyrosine as substrate; N.D., not detected. Data are expressed as means ± S.D. (n = 3). ***, $p \leq$

0.001. (B)Analysis of phenylpyruvic acid content in per gram of fresh *N. benthamiana* leaves. Vector, empty vector GV3101 overexpressed in *N. benthamiana*. CsAAAT1 and CsAAAT2 represent CsAAAT1 and CsAAAT2 overexpressed in *N. benthamiana*, respectively. Data are expressed as means ± S.D. (n = 5). Columns with different letters above are significantly different (Duncan's test, $p \leq 0.05$; one-way ANOVA). (C) Subcellular localization analyses of CsAAAT1-GFP and CsAAAT2-GFP. Upper photos: CsAAAT1-GFP in cytoplasm of tobacco leaf cell. Lower photos: CsAAAT2-GFP in cytoplasm of tobacco leaf cell. Green and red panels show GFP fluorescence and chloroplast auto-fluorescence, respectively. Merged panel shows overlay of green and red fluorescence images.

Figure 6. Proposed biosynthesis pathway of benzaldehyde, benzyl alcohol, and methyl benzoate from L-phenylalanine *via* phenylpyruvic acid in tea leaves.

L-Phe, L-phenylalanine; PPA, phenylpyruvic acid; CsAAAT1, *Camellia sinensis* aromatic amino acid transaminase 1; BAld, benzaldehyde; BAlc, benzyl alcohol; MeBA, methyl benzoate.

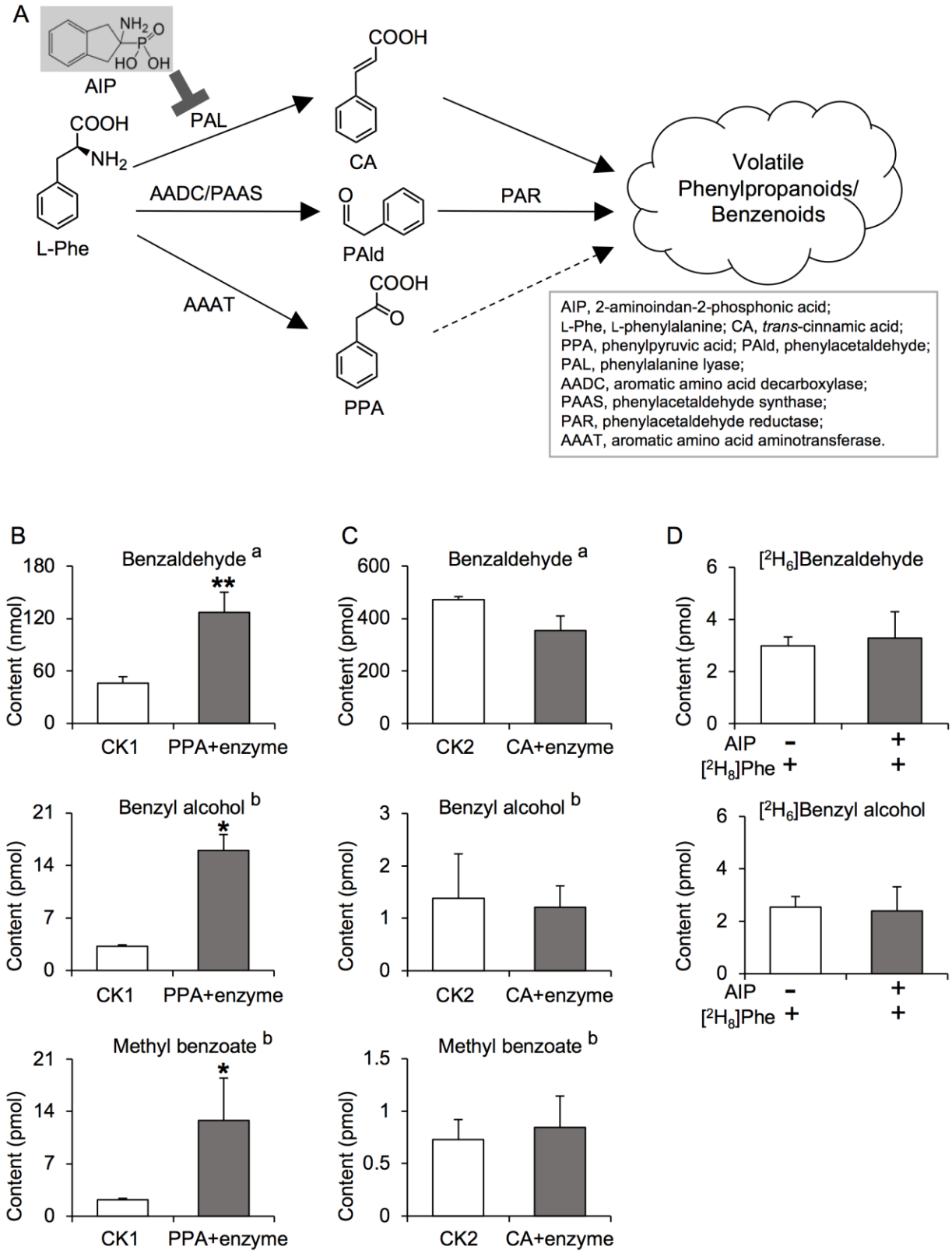
A
AIP
PAL
COOH
NH2
L-Phe
CA
AADC/PAAS
PAld
PAR
Volatile Phenylpropanoids/ Benzenoids
AAAT
PPA
AIP, 2-aminoindan-2-phosphonic acid;
L-Phe, L-phenylalanine; CA, trans-cinnamic acid;
PPA, phenylpyruvic acid; PAld, phenylacetaldehyde;
PAL, phenylalanine lyase;
AADC, aromatic amino acid decarboxylase;
PAAS, phenylacetaldehyde synthase;
PAR, phenylacetaldehyde reductase;
AAAT, aromatic amino acid aminotransferase.
B
Benzaldehyde a
Content (nmol)
CK1
PPA+enzyme
Benzyl alcohol b
Content (pmol)
Methyl benzoate b
C
Benzaldehyde a
CK2
CA+enzyme
Benzyl alcohol b
Methyl benzoate b
D
[2H6]Benzaldehyde
AIP
[2H8]Phe
[2H6]Benzyl alcohol

Figure 1

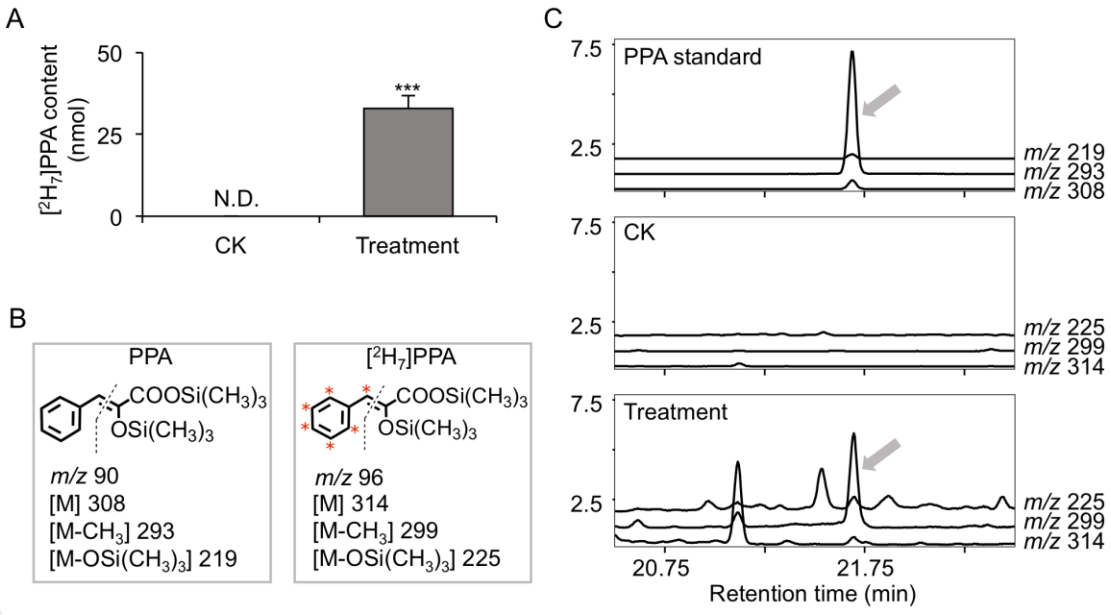
A
[2H7]PPA content (nmol)
50
25
0
***
N.D.
CK
Treatment
B
PPA
COOSi(CH3)3
OSi(CH3)3
m/z 90
[M] 308
[M-CH3] 293
[M-OSi(CH3)3] 219
[2H7]PPA
COOSi(CH3)3
OSi(CH3)3
m/z 96
[M] 314
[M-CH3] 299
[M-OSi(CH3)3] 225
C
PPA standard
7.5
2.5
m/z 219
m/z 293
m/z 308
CK
m/z 225
m/z 299
m/z 314
Treatment
20.75
21.75
Retention time (min)

Figure 2

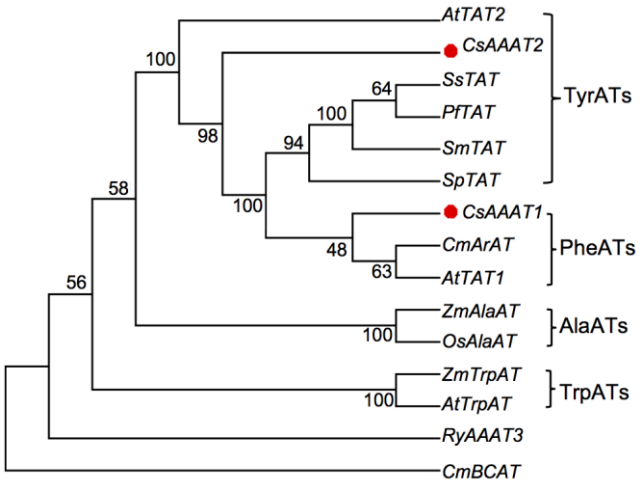
AtTAT2
CsAAAT2
SsTAT
PfTAT
SmTAT
SpTAT
CsAAAT1
CmArAT
AtTAT1
ZmAlaAT
OsAlaAT
ZmTrpAT
AtTrpAT
RyAAAT3
CmBCAT
TyrATs
PheATs
AlaATs
TrpATs
100
64
100
98
94
58
100
48
63
56
100
100

Figure 3

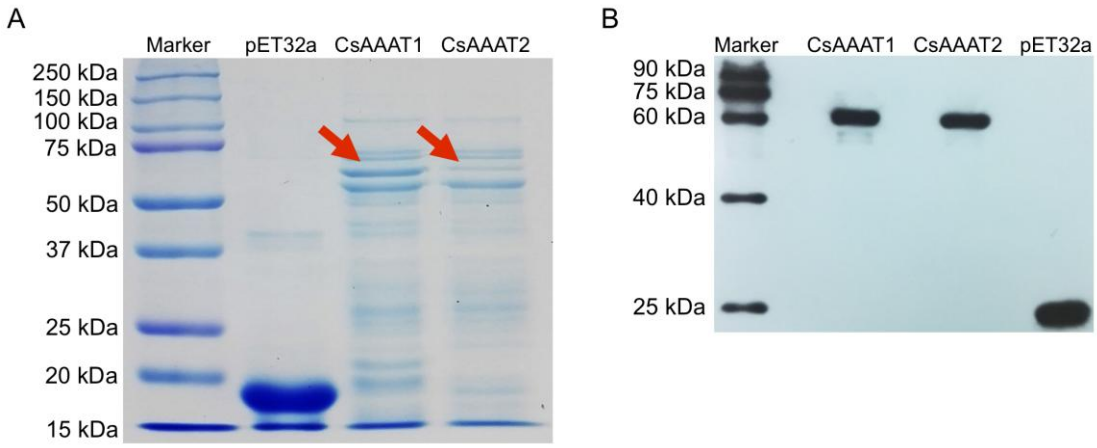
A
Marker
pET32a
CsAAAT1
CsAAAT2
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
B
Marker
CsAAAT1
CsAAAT2
pET32a
90 kDa
75 kDa
60 kDa
40 kDa
25 kDa

Figure 4

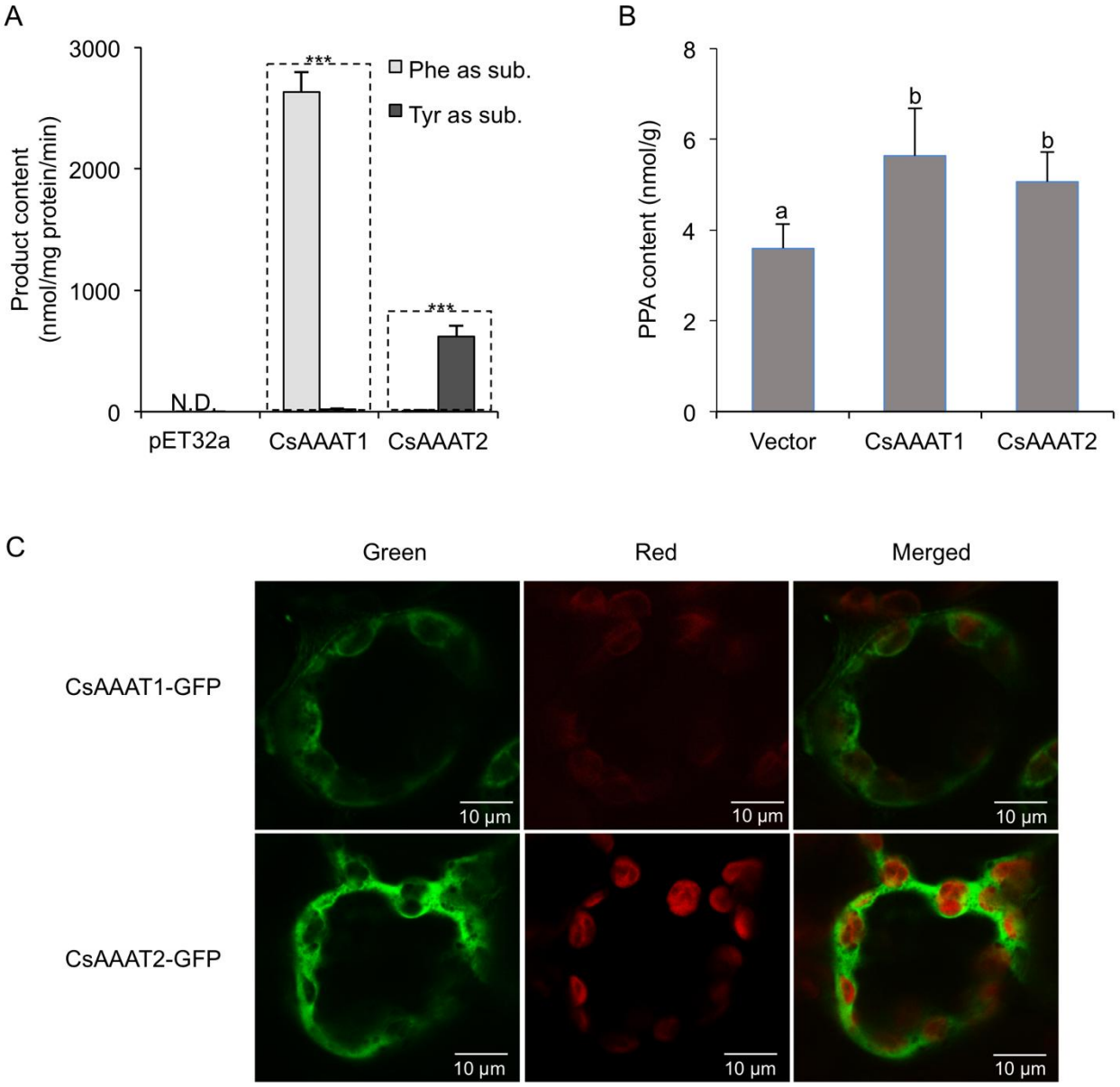
A
3000
2000
1000
0
Product content (nmol/mg protein/min)
***
***
N.D.
Phe as sub.
Tyr as sub.
pET32a
CsAAAT1
CsAAAT2
B
8
6
4
2
0
PPA content (nmol/g)
a
b
b
Vector
CsAAAT1
CsAAAT2
C
Green
Red
Merged
CsAAAT1-GFP
CsAAAT2-GFP
10 μm

Figure 5

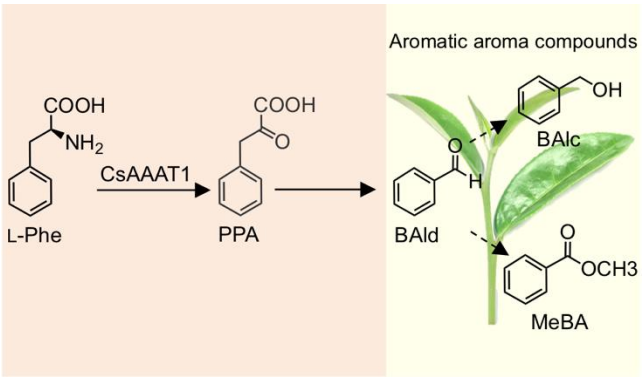
Aromatic aroma compounds
COOH
NH2
L-Phe
CsAAAT1
COOH
O
PPA
OH
BAlc
O
H
BAld
O
OCH3
MeBA

Figure 6

Highlights

► Phenylpyruvic acid was the precursor of benzaldehyde in tea.

► Phenylpyruvic acid was derived from L-phenylalanine in tea.

► CsAAAT1 showed high L-phenylalanine transamination activity.

► CsAAAT1 was localized in the cytoplasm of leaf cells.