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Studies on the Biochemical Formation Pathway of the Amino Acid L-Theanine in Tea (*Camellia sinensis*) and Other Plants

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Supporting Information

ABSTRACT: Tea (*Camellia sinensis*) is the most widely consumed beverage aside from water. The flavor of tea is conferred by certain metabolites, especially L-theanine, in *C. sinensis*. To determine why more L-theanine accumulates in *C. sinensis* than in other plants, we compare L-theanine contents between *C. sinensis* and other plant species (*Camellia nitidissima*, *Camellia japonica*, *Zea mays*, *Arabidopsis thaliana*, and *Solanum lycopersicum*) and use a stable isotope labeling approach to elucidate its biosynthetic route. We quantify relevant intermediates and metabolites by mass spectrometry. L-Glutamic acid, a precursor of L-theanine, is present in most plants, while ethylamine, another precursor of L-theanine, specifically accumulates in *Camellia* species, especially *C. sinensis*. Most plants contain the enzyme/gene catalyzing the conversion of ethylamine and L-glutamic acid to L-theanine. After supplementation with [$^2H_5$]ethylamine, all the plants produce [$^2H_5$]L-theanine, which suggests that ethylamine availability is the reason for the difference in L-theanine accumulation between *C. sinensis* and other plants.

KEYWORDS: *alanine, amino acid, Camellia sinensis, ethylamine, stable isotope labeling, tea, theanine*

INTRODUCTION

Next to water, tea (*Camellia sinensis*) is the most popular beverage worldwide. Numerous studies have validated the health benefits of tea, and consumers appreciate the unique taste and aroma.[1] In contrast to other plants, *C. sinensis* produces specialized metabolites that determine the functions and quality attributes of tea. It is an interesting and important scientific question to investigate why these specialized metabolites accumulate in *C. sinensis*, but barely accumulate in other plant species. Theanine (γ-glutamylethylamine) is one of the specialized metabolites present in *C. sinensis*. Like other natural amino acids, theanine occurs in its L-enantiomer form in nature. L-Theanine is a unique amino acid in *C. sinensis* leaves that has a special umami flavor, which confers the special taste of green tea.[2,3] L-Theanine has many biological effects on humans, including promoting relaxation, concentration, and the ability to learn. It also has neuroprotective effects, cooperative effects with antitumor agents, protects against vascular diseases, counteracts the effects of caffeine, and has antiobesity effects.[4−11] Besides its contribution to the function and quality of tea, L-theanine plays important physiological roles in plants. Amino acids are not only the basic units for protein synthesis, but also serve as an energy source and as precursors for the biosynthesis of many bioactive molecules.[12] Among them, L-glutamic acid (Glu) and L-glutamine (Gln) are two crucial amino acids that contribute to the nitrogen cycle. In general, plants assimilate nitrogen via the synthesis of Glu and Gln.[13] L-Theanine is a Gln analog and shares many of its properties. Similar to Gln, L-theanine is involved in ammonia detoxification via the conversion of toxic ammonia into the nontoxic amino acid form.[14−16] As L-theanine accounts for more than 50% of total amino acids of *C. sinensis* plants, it fixes a similar amount of nitrogen as do Glu and Gln combined.[17]

Since L-theanine has important functions in humans and plants, its distribution and biosynthesis in plants have been topics of interest. A wide variety of *Camellia* species contain L-theanine, and it is especially abundant in *C. sinensis*, which is used to produce tea.[18] A few *Ericales* plants and the edible mushroom *Xerocomus badius* also contain L-theanine.[14,19] In *C. sinensis* plants, L-theanine is biosynthesized by L-theanine synthetase (TS, EC 6.3.1.6) from Glu and ethylamine.[20] As L-theanine is very similar to Gln, TS is highly homologous to Gln synthetase (GS, EC 6.3.1.2). A previous study reported that *CsTS1* shows 99% homology to *CsGS1.3*, and *CsTS2* shows 97% to *CsGS1.1*.[21] Wu et al.[22] identified 20 *CsGS* genes, including several unconfirmed *CsTSs*. Studies on bacteria, *C. sinensis*, and pea seeds have shown that GS can convert Glu and free ammonia to Gln, and also transfer Glu to L-theanine in the presence of ethylamine.[20,23−26] These findings suggest that GS and TS are very similar not only in their DNA sequences, but also in their enzymatic activities. As GS/TS are present in many plants, it is still unknown why L-theanine accumulates only in *C. sinensis*, and barely or not at all in other plant species.

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To answer this question, we determined the contents of L-theanine and its related metabolites (ethylamine, Glu, and L-alanine) in two model plant species (*Arabidopsis thaliana* and *Solanum lycopersicum*), the economic plant *Zea mays*, and two *Camellia* species (*Camellia nitidissima* and *Camellia japonica*), and compared them with those in *C. sinensis*. Then stable isotope-labeled ethylamine was supplied to each species to investigate the pathway leading to the specific accumulation of L-theanine. The results of this study help to explain why *C. sinensis* accumulates L-theanine, which determines the quality of tea products, and will be useful for breeding and screening high-L-theanine lines of *C. sinensis*.

MATERIALS AND METHODS

Chemicals. Amino acid standard solutions for the amino acid analyzer were purchased from SYKAM GmbH, Eresing, Germany. Amino acid authentic standards for mass analysis were purchased from Chengdu Mansite Pharmacetical Co., Ltd., Chengdu, China. *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Regis Technoloogies Inc., Morton Grove, USA. Ethylamine:HCl was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China. [$^2H_5$]Ethylamine:HCl ($^2H_5$% = 98%) was purchased from Cambridge Isotope Laboratories Inc., MA, USA. Isobutyl chloroformate (IBCF) was purchased from Shanghai Macklin Biochemical Co., Ltd., Shanghai, China. Isopropyl-β-D-thiogalacto-pyranoside (IPTG) was purchased from Sigma-Aldrich Company Ltd., USA. Coomassie Blue R250, 30% acrylamide/bis solution, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEMD), and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad Laboratories, California, USA. Ni-NTA resin was purchased from Qiagen Inc., Hilden, Germany. PD-10 desalting column was purchased from GE Healthcare Life Sciences, Chicago, USA.

Plant Materials and Soils. The roots and leaves of *A. thaliana*, *S. lycopersicum* cv. Micro-Tom, *Z. mays* var. rugosa Bomaf., *C. nitidissima*, *C. japonica*, and *C. sinensis* var. Jinxuan were used for the present study. The seeds of *A. thaliana* were scattered on the wet filter paper under 4 °C for 3 days. Afterward, these *A. thaliana* seeds were dispersed 4−6 per pot. The seedlings of *A. thaliana* were transferred for one seedling per pot after budding. The seeds of *S. lycopersicum* and *Z. mays* were sown one per pot. The seeds of *A. thaliana*, *S. lycopersicum*, and *Z. mays* were grown in a growth chamber controlled at 25 °C humidity 80%, under 16-h light and 8-h dark cycles and were watered one time per 3 days before budding and one time per week after budding. The plants of *C. nitidissima*, *C. japonica*, and *C. sinensis* were grown in fields. The soils for cultivating *A. thaliana*, *S. lycopersicum*, and *Z. mays* were purchased from Treffex AS, Pärnumaa, Estonia (producer) on behalf of Jiffy Products International BV, Moerdijk, Netherlands. The soils for cultivating each plant species were also collected for analyses. All the samples were collected in October, 2016.

Analysis of L-Alanine, L-Theanine, and Glu by an Amino Acid Analyzer. The extraction protocol and analysis method of free amino acids were described by the previous studies.[27−29] Plant tissues (200 mg fresh weight, finely powdered) were extracted with 0.7 mL of cold methanol (100%) by vortexing for 2 min followed by ultrasonic extraction in ice cold water for 20 min. The extracts were mixed with 0.7 mL of chloroform and 0.2 mL of cold water for phase separation. The resulting upper layer was dried and used as the crude extract of amino acids. For analysis of all the free amino acids, the dried crude extract was dissolved in 1 mL of 5% sulfosalicylic acid and stood for 1 h. After being centrifuged at 12 000 × *g* for 10 min, the supernatant was filtered through a 0.45 μm membrane and subjected to an amino acid analyzer. A Sykam S433D Physiological Li C4 system (SYKAM GmbH, Eresing, Germany) equipped with quaternary pump, column oven, refrigerated auto sampler, and UV−vis detector was used. The Physiological Li C4 system was coupled with S4300 postcolumn derivatization system. The experiments were performed on a high efficiency sodium cation-exchange Pickering Laboratories column (4.0 × 150 mm^2). The Sykam S433D Physiological Li C4 system was operated using a mobile phase consisting of lithium citrate pH 2.9, pH 4.2, pH 8.0, and using UV−vis detection at 570 and 440 nm. The flow-rate of the mobile phase was 0.45 mL/min, and the flow-rate of the derivatizating reagent was 0.25 mL/min. The column temperature was set at 38 °C, and the post column reaction equipment was kept at 130 °C temperature. The temperature of the autosampler was kept at 5 °C, and the injection volume was 50 μL for both standard and samples. The areas under the peaks corresponding to the amounts of amino acids were compared to the authentic standards of amino acids.

Analyses of Gln by Gas Chromatography−Mass Spectrometry (GC−MS). The MSTFA derivatization and GC−MS were employed to analyze Gln.[29] The crude extract of amino acids as described above was dissolved in 100 μL of pyridine and derivatized with 50 μL of MSTFA at 37 °C for 60 min, cooled, and then centrifuged. The MSTFA derivates were then analyzed by a GC−MS QP2010 SE (Shimadzu Corporation, Kyoto, Japan). The injector temperature was 240 °C, splitless mode was used with a splitless time of 1 min, and helium was the carrier gas with a velocity of 1.0 mL/min. An HP-5 column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, California, USA) was used with an initial temperature of 100 °C for 2 min, a ramp of 5 °C/min to 300 °C, and then a hold at 300 °C for 10 min. The MS was operated with selective ion monitoring mode (*m*/*z* 156 and 245 for Gln-MSTFA derivate).

Analysis of Ethylamine by GC−MS. The extraction protocol of internal ethylamine in fresh samples was described by Tsushida and Takeo.[30] Fresh samples were steamed for 60 s and immediately frozen at −20 °C. Then the frozen samples were lyophilized and pulverized. Powdered samples (100 mg) were put into a 10 mL volumetric flask and diluted with 5 mL of distilled water. The flask was warmed at 80 °C for 5 min and allowed to stand for 2 h at room temperature. Then the extract was filtered and centrifugated at 3500 × *g* for 5 min.

The analytical method of ethylamine was described by Almeida et al.[31] with a modification.

An aliquot of sample (1 mL) was transferred to a 4 mL silanized screw-capped glass vial containing 1 mL of toluene, and the mixture was adjusted to alkaline using 1 mL of 0.5 M phosphate buffer (pH 12.0), and 25 μL of IBCF and a deuterated internal standard (IS) [$^2H_5$]ethylamine:HCl (2 mg mL^{-1}) were added. The vial was shaken for 10 min at 250 rpm and centrifugated at 3500 × *g* for 5 min. The resultant toluene (upper) layer (200 μL) was transferred to another vial, and 200 μL of alkaline methanol, which prepared by dissolving KOH in methanol until saturation and followed by filtration through a 0.45 μm filter, was added. The tube was shaken for 5 min, then 600 μL of 5 M NaOH was added, and the mixture was shaken for another 5 min. After the mixture was centrifugated, the toluene layer was used for analyzing ethylamine. The ICBF derivatizated products were analyzed by the GC−MS. GC separation was performed on an HP-5 column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, California, USA). The injector temperature was 280 °C, splitless mode was used with a splitless time of 1 min, and helium was the carrier gas with a velocity of 1.0 mL/min. The oven temperature program was as follows: 40 °C held for 3.0 min, ramped to 140 °C at 40 °C/min, then ramped to 280 °C at 80 °C/min, and held for 10 min. The MS was operated with selective ion monitoring mode (*m*/*z* 90 and 72 for ICBF-ethylamine derivate).

Supplement of [$^2H_5$]Ethylamine to Different Plants and Identification of [$^2H_5$]L-Theanine by Ultra-Performance Liquid Chromatography−Quadrupole Time-of-Flight Mass Spectrometry (UPLC−QTOF-MS). The roots and shoots obtained from plant materials mentioned above were placed in tubes with 2 mL of distilled water containing [$^2H_5$]ethylamine (2 mg) and blank as controls. The tubes were incubated at 25 °C for 24 h. Then samples were harvested, frozen in liquid N_2 immediately, and extracted by the protocol mentioned above. *O*-Phthalaldehyde (OPA, 25 μL) derivation buffer, which added 50 μL of OPA alcohol solution and 25 μL of β-mercaptoethanol into 450 μL of 3% boric acid buffer (pH 10.5), was added into 300 μL of sample solution and stood at 25 °C for 10 min. Afterward, 25 μL of 0.1 M acetic acid was added to end the reaction, and then 200 μL of distilled water was added. After centrifugation, the resultant supernatant was filtered through a

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0.45 μm membrane and subjected to an UPLC−QTOF-MS (Acquity UPLC I-Class/Xevo G2-XS QTOF, Waters Corporation, MA, USA). Each sample (5 μL) was injected onto a Waters ACQUITY UPLC C_{18} column (1.7 μm, 2.1 mm × 100 mm, Waters Corporation, MA, USA). Solvent A was water with 0.1% (v/v) formic acid. Solvent B was acetonitrile with 0.1% (v/v) formic acid. The flow rate was set at 0.3 mL/min with a gradient elution, which started at 76% of solvent A and kept for 8 min, then decreased linearly to 10% in 0.1 min and kept for 4 min, and raised up to 76% in 0.1 min and kept for 4 min. The column was maintained at 40 °C, and the samples were maintained at a temperature of 4 °C prior to injection. The electrospray ionization used a capillary voltage of 3.0 kV for positive mode; cone voltage 50 V; source temperature 100 °C; desolvation temperature 350 °C; cone gas flow 50 L/h; and desolvation gas flow 650 L/h. The scan range was m/z 50−1000 Da and the mass spectrometer resolution was 30 000, which enabled mass accuracy within 10 mDa. The Leu-enkephalin (m/z 556.2771) was used as the lock mass solution for accurate mass calibration during long analytical sequences to counteract the potential effect of calibration drift during the long analytical run time. The characteristic m/z values ($[M + H]^+$) of OPA derivatizated products of $[^2H_5]$L-theanine and L-theanine were 356.1692 and 351.1379, respectively.

Phylogenetic Analyses of CsTSs and GS/TS from Other Plants. A similarity search for all the unique sequences was carried on National Center for Biotechnology Information (NCBI) database. Sequences were aligned using the ClustalX program. The phylogenetic tree was calculated using neighbor-joining algorithms and generated by MEGA 5 software. Bootstrap analysis of the NJ tree was performed using 1000 replicates

AtGSs Recombinant Expression and Function Identification. *AtGS1* was amplified using primers (Table S1) with an *Eco*RI site (bold) in the forward primers and a *Sal*I site (bold) in the reverse primers; *AtGS2* was amplified using primers (Table S1) with an *Eco*RI site (bold) in the forward primers and a *Xho*I site (bold) in the reverse primers. The above amplified cDNAs were subcloned into the corresponding sites of digested pET32a plasmid and renamed pET32a-AtGS1 and pET32a-AtGS2, respectively. After verification by sequencing, the expression construct was transformed into *E. coli* Rosetta for recombinant protein expression. A single colony from each transformed *E. coli* Rosetta cell line was incubated overnight at 37 °C in 1 mL of LB containing ampicillin, chloramphenicol, and kanamycin at a concentration of 100 μg/mL, 34 μg/mL, and 15 μg/mL, respectively. Inocula were then individually added to 200 mL of LB, containing 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, and 15 μg/mL kanamycin, and grown at 37 °C to an OD_{600} at 0.6. After addition of 0.1 mM IPTG, the cultures were grown at 20 °C for another 16 h to produce recombinant protein. Cells were harvested at 10 000 × g for 10 min, then disrupted by sonication for 20 min in a 50 mM NaH_2PO_4 (pH 8.0) buffer containing 300 mM NaCl and 10 mM imidazole. After centrifugation at 10 000 × g for 20 min, the supernatant was collected and purified by using affinity binding on Ni Sepharose 6 Fast Flow (GE Healthcare Life Sciences, Chicago, USA) according to the manufacturer's instruction. The purified protein was passed through a PD-10 (GE Healthcare Life Sciences, Chicago, USA) desalting column. The protein samples were diluted 1:2 with SDS loading buffer and denatured for 10 min at 100 °C. SDS-PAGE was performed using 10% (w/v) polyacrylamide gels.

Reaction solution (total volume 800 μL) for L-theanine synthesis activity contained 25 μmol of Glu, 10 μmol of ethylamine:HCl, 25 μmol of β-mercaptothanol, 25 μmol of $MgCl_2$, 100 μmol of Tris-HCl (pH 7.5), 3 μmol of K_3PO_4, 25 μmol of KOH, 10 μmol of ATP, and 100 μL of AtGSs recombinant protein supernatant (50 μg).[20,32] The reaction mixture was incubated at 30 °C for 2 h, terminated by heating at 96 °C for 2 min, and filtrated through a PD-10 (GE Healthcare Life Sciences, Chicago, USA) desalting column. The reaction solution for Gln synthesis activity contained the same compounds but ethylamine replaced with ammonium. After drying, the L-theanine and Gln products were detected by MSTFA derivatization and GC−MS analysis as described above.

Table 1. Contents of L-Theanine, L-Glutamine, L-Glutamic Acid, L-Alanine, and Ethylamine in Roots and Leaves of Different Plants[a]

	Camellia sinensis		*Camellia japonica*		*Camellia nitidissima*		*Arabidopsis thaliana*		*Zea mays*		*Solanum lycopersicum*	
	root	leaf	root	leaf	root	leaf	root	leaf	root	leaf	root	leaf
L-theanine (mg g^{-1})	16.43 ± 1.52	1.96 ± 0.76	0.31 ± 0.01	0.31 ± 0.09	0.89 ± 0.04	0.53 ± 0.02	N.D.	N.D.	trace	trace	N.D.	N.D.
L-glutamine (mg g^{-1})	0.75 ± 0.17	0.29 ± 0.06	0.12 ± 0.01	0.05 ± 0.01	0.20 ± 0.03	0.01 ± 0.005	0.24 ± 0.09	10.25 ± 1.83	0.07 ± 0.01	0.48 ± 0.09	0.58 ± 0.18	0.63 ± 0.22
L-glutamic acid (mg g^{-1})	0.30 ± 0.01	0.40 ± 0.03	0.37 ± 0.05	0.43 ± 0.09	0.93 ± 0.04	0.60 ± 0.02	1.37 ± 0.06	2.79 ± 0.18	0.07 ± 0.02	1.80 ± 0.08	1.01 ± 0.38	2.24 ± 1.24
L-alanine (mg g^{-1})	0.28 ± 0.04	0.07 ± 0.02	0.04 ± 0.02	0.05 ± 0.01	0.27 ± 0.06	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.03 ± 0.01	0.87 ± 0.55	0.11 ± 0.01	0.13 ± 0.04
ethylamine (μg g^{-1})	420.44 ± 100.98	31.50 ± 3.56	4.39 ± 0.27	1.58 ± 0.53	1.86 ± 0.77	1.08 ± 0.01	N.D.	N.D.	8.05 ± 0.87	5.32 ± 0.49	N.D.	N.D.

[a]Data are expressed as mean ± SD (n = 3). N.D., not detected.

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RESULTS AND DISCUSSION

Ethylamine Availability Explains the Difference in L-Theanine Accumulation between *C. sinensis* and Other Plants. First, we quantified L-theanine in roots and leaves of *C. sinensis* *C. nitidissima*, *C. japonica*, *Z. mays*, *A. thaliana*, and *S. lycopersicum*. L-Theanine specifically accumulated in *Camellia* plants, especially *C. sinensis*, and also occurred in *Z. mays* with tract amount. Furthermore, in *Camellia* plants, the L-theanine content was higher in the roots than in the leaves (Table 1). Because Glu and ethylamine are the precursors of L-theanine in *C. sinensis*, we also quantified these compounds. All of the tested species contained Glu, but only *Z. mays* and *Camellia* species, especially *C. sinensis*, accumulated ethylamine (Table 1). In the plant species containing ethylamine, the ethylamine content was higher in the roots than in the leaves (Table 1). Deng et al. investigated the L-theanine content in the leaves of 27 species or varieties of Theaceae plants. They found that 21 species or varieties contained L-theanine, but at much lower levels than those in *C. sinensis*. Ethylamine and Glu, the substrates of TS, were found to be distributed almost uniformly among all tested parts of the seedlings.[33] In our study, we quantified L-theanine and its related metabolites in plant species that are not in the Theaceae and found that all of the plants containing L-theanine also contained ethylamine. Since L-theanine is a unique amino acid in *C. sinensis*, it was paid more attention in Theaceae plants and less in others. Both L-theanine and ethylamine were detected in *Z. mays* (Table 1), which indicated that *Z. mays* could also transform ethylamine to L-theanine. To investigate whether ethylamine is the restricting factor for the differential accumulation of L-theanine between *C. sinensis* and other plants, we conducted stable isotope-labeling experiments (Figure 1A). After [$^2H_5$]ethylamine was supplied to the roots and leaves of *C. sinensis*, *C. nitidissima*, *C. japonica*, *Z. mays*, *A. thaliana*, and *S. lycopersicum*, [$^2H_5$]L-theanine was detected in all plants, even including *A. thaliana* and *S. lycopersicum*, which did not contain endogenous ethylamine (Figure 1B,C). Tracer experiments in plants with isotope-labeled precursors have been used to discover biochemical pathways of secondary metabolites in several studies.[34−36] In fact, L-theanine was found to be derived from ethylamine in *C. sinensis* by using ^{14}C-labeled ethylamine.[34] A previous study showed that L-theanine is produced from ethylamine in several different tissues of *C. sinensis* (cotyledons, shoots, and roots).[34] In the present study, isotope-labeled ethylamine was converted into labeled L-theanine in *C. nitidissima*, *C. japonica*, *Z. mays*, *A. thaliana*, and *S. lycopersicum*. This finding indicates that the enzymes/genes involved in the pathway leading from ethylamine and Glu to L-theanine are present in a wide variety of plants.

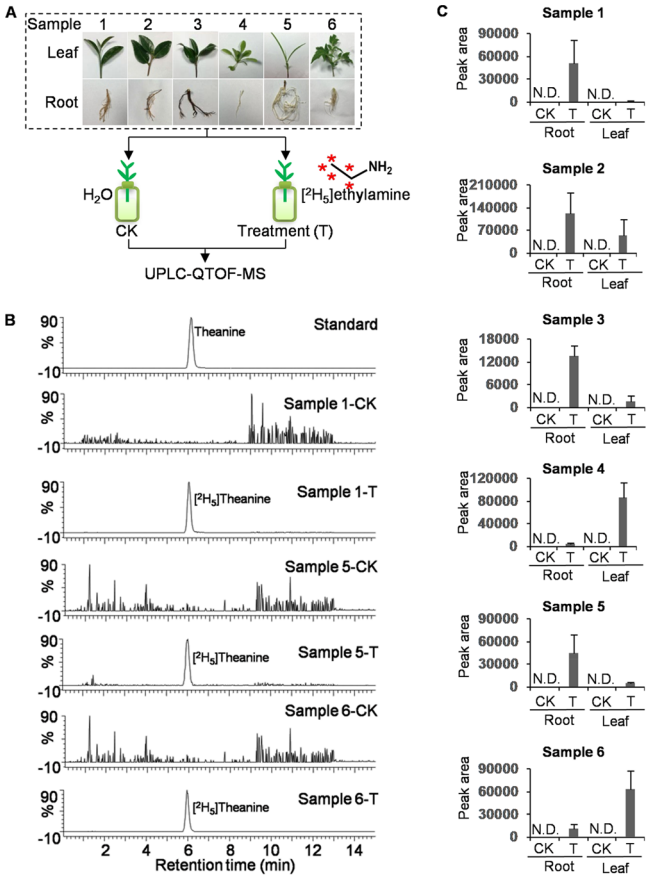

Figure 1. Supplement of [$^2H_5$]ethylamine to different plants and identification of [$^2H_5$]L-UPLC−QTOF-MS. CK, H_2O treatment. Treatment (T), [$^2H_5$]ethylamine treatment. (A) Leaf and root of the selected plants were treated with [$^2H_5$]ethylamine and then analyzed by UPLC−QTOF-MS. 1, *C. sinensis*; 2, *C. japonica*; 3, *C. nitidissima*; 4, *Arabidopsis thaliana*; 5, *Zea mays*; 6, *Solanum lycopersicum*. (B) UPLC−QTOF-MS analyses of authentic standard of theanine and [$^2H_5$]theanine in *C. sinensis* (sample 1), *Zea mays* (sample 5), and *Solanum lycopersicum* (sample 6) treated with [$^2H_5$]ethylamine. The characteristic m/z values ([M + H]$^+$) of OPA derivatizated products of [$^2H_5$]theanine and theanine were 356.1692 and 351.1379, respectively. (C) UPLC−QTOF-MS peak areas of [$^2H_5$]theanine in leaves and roots of the selected plants treated with [$^2H_5$]ethylamine. N.D., not detected. Data are expressed as mean ± SD (n = 3).

L-Theanine Synthetases in Plants Other than *C. sinensis*. To investigate whether TSs are also present in other plants, GSs/TSs from *A. thaliana* were isolated, cloned, sequenced, and functionally characterized. The phylogenetic analysis (Figure 2) revealed that the amino acid sequence of CsTS1/2 showed high homology with sequences from *Arabidopsis*, tomato, and maize, while *GS1* and *GS2* were closely clustered. The complete open reading frames of *AtGS/TS1* and *AtGS/TS2* contained 1071 nucleotides with a calculated protein molecular mass of 39 kDa, and 1293 nucleotides with a calculated protein molecular mass of 47 kDa, respectively (Figure 3).

The activity of TS was evaluated using a homogenate of 11-day-old etiolated tea seedlings from which the cotyledons had been removed. The crude tea TS preparation was very labile and contained ATP-hydrolyzing enzymes. Therefore, a creatine phosphate and creatine phosphokinase system was required to generate ATP.[37] The use of this system and fractionation with acetone, ammonium sulfate, and sephadex G-50 provided a much more stable enzyme preparation than the crude tea homogenate. The optimum pH of the enzyme is known to be 7.5.[20] In another study, TS activity was detected in acetone powder extracts of imbibed pea seeds. The TS activity of the pea seed enzyme was substantially inhibited by ammonia at low concentrations (77% inhibition with 0.5 mM ammonia). Therefore, L-theanine synthesis by pea enzyme preparations was ascribed to a nonspecific reaction of GS. In contrast, the tea TS was shown to be a specific enzyme for L-theanine synthesis from ethylamine.[23] Since Sasaoka et al. first reported the activity of native TS,[20] only a few publications have reported on this enzyme. Li et al. determined the activity of TS obtained from tea seedlings using capillary electrophoresis with 2,4-dinitrofluorobenzene as a derivative reagent, but these analyses could not provide information on the properties of the enzyme.[32]

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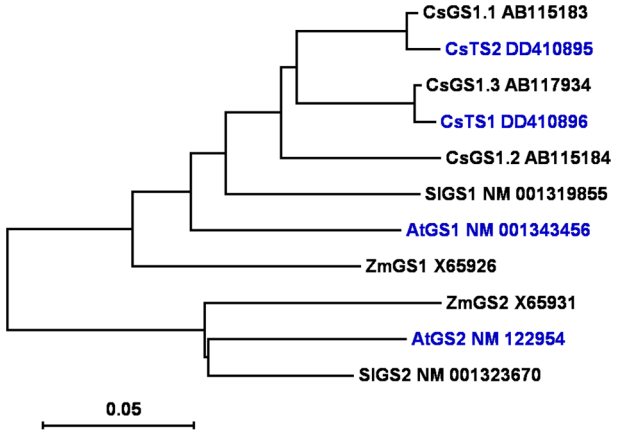

Figure 2. Phylogenetic analysis of CsGS/TSs and related functionally characterized GSs. *At, A. thaliana*; *Zm, Zea mays*; *Sl, S. lycopersicum*; *Cs, C. sinensis*. TS, theanine synthetase; GS, glutamine synthetase. Sequences were aligned using the ClustalX program. The neighbor-joining phylogenetic tree was generated by MEGA 5 software. The proteins included in the tree are represented by GenBank accession number.

Attempts to purify the native enzyme to apparent homogeneity have not yet been successful, probably because the enzyme activity is low and labile in tea extracts.

The TaiyoKagaku Company has a patent on *TS* genes.[38] Two genes encoding TS isoforms, TSl (DD410896) and TS2 (DD410895), have been cloned from the cDNA library of tea. In a previous study, transcripts of *CsTS1* and *CsTS2* were found in all organs, but at lower levels in cotyledons than in roots and shoots.[21] The native *CsGSs* have also been cloned and characterized.[39] Although TS activity has been studied using partially purified enzyme preparations and its encoding genes have been patented, it is still unclear if the reported *CsTS1* and *CsTS2* are distinct from *CsGSs*.[14] We analyzed all the *CsGS* sequences in the NCBI, and they were homologous to *CsGS1.1* (GenBank accession no. AB115183), *CsGS1.2* (GenBank accession no. AB115184), and *CsGS1.3* (GenBank accession no. AB117934). The *CsTS* sequences were the same as *CsTS1* (DD410896) and *CsTS2* (DD410895). *CsTS1* shared 99% sequences of *CsGS1.3*, and *CsTS2* shared 97% of *CsGS1.1*. The genes encoding GSs have been cloned and characterized from *Arabidopsis*,[40] *Z. mays*,[41,42] and *S. lycopersicum*.[43] These GSs

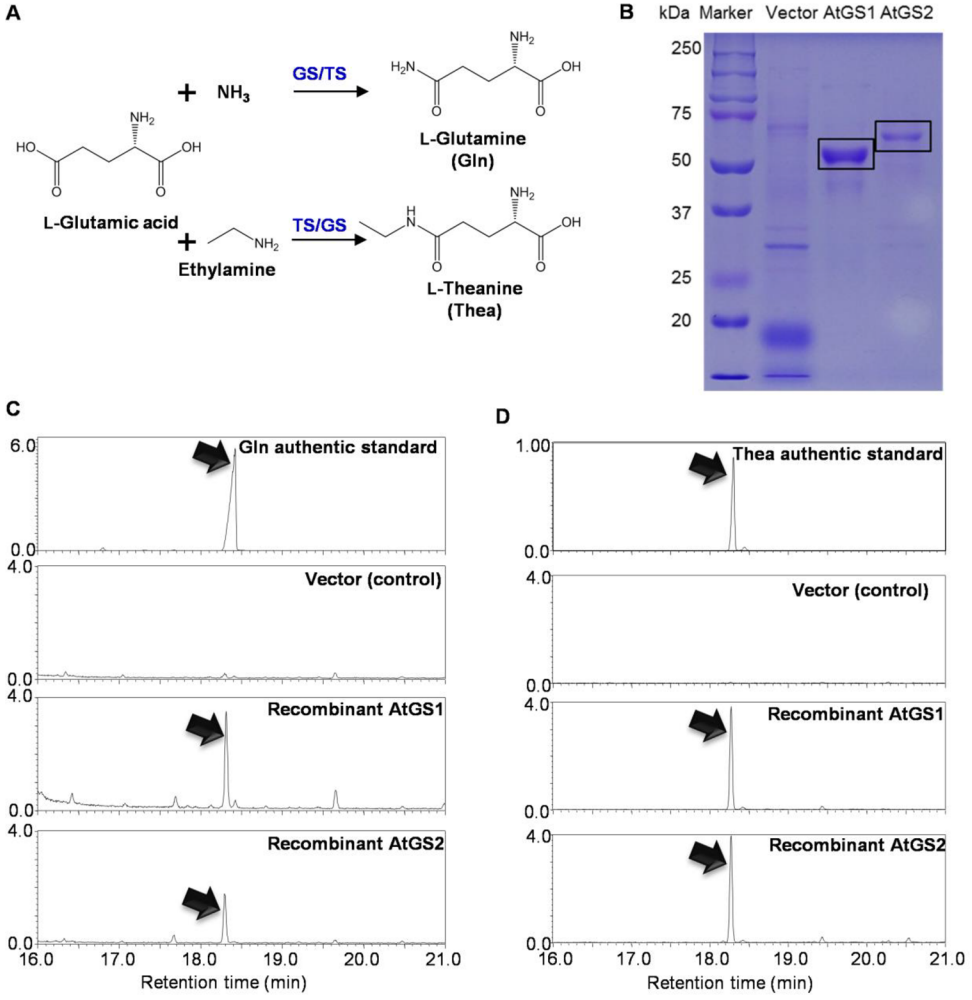

Figure 3. Biosynthetic pathways of L-glutamine and L-theanine, and identification of recombinant AtGSs. (A) Biosynthetic pathways of L-glutamine and L-theanine. (B) SDS-PAGE analysis of AtGSs expressed in *E. coli*. Black bold frames indicate target proteins. Proteins were resolved by SDS-PAGE on polyacrylamide gel and stained with Coomassie brilliant blue. Purified His-tagged combined AtGS1 and AtGS2 with a calculated protein molecular mass of about 57 kDa and about 66 kDa, respectively. (C) GC−MS identification of L-glutamine derived from L-glutamic acid and ammonia under action of recombinant AtGS1 and AtGS2. *m/z* 156 for L-glutamine-MSTFA derivate. (D) GC−MS identification of L-theanine derived from L-glutamic acid and ethylamine under action of recombinant AtGS1 and AtGS2. *m/z* 159 and 273 for L-theanine-MSTFA derivate.

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catalyze the conversion of Glu and free ammonium to Gln and are involved in nitrogen metabolism. However, it is still unknown whether these GSs catalyze the conversion of Glu and ethylamine to L-theanine. The present study showed that GSs from *Arabidopsis* were able to produce Gln and L-theanine (Figure 3), which indicated that the GSs in a wide variety of plant species can synthesize L-theanine if the right substrate is present.

Formation of Ethylamine. The results of this study indicate that ethylamine availability is the restricting factor for the differential accumulation of L-theanine between *C. sinensis* and other plant species. This led us to investigate why *C. sinensis* accumulates ethylamine and other plants do not. Ethylamine may be derived from soil or it may be synthesized by the plant. No ethylamine was detected in the soils used to cultivate the plants in this study (Figure S1), which suggested that ethylamine is synthesized by the plants themselves.

Amines are compounds with aromatic and aliphatic structures that are widespread in the plant kingdom. They form during metabolism and show diverse characteristics and biological functions.[44] Ethylamine occurs in *Camellia*, *Vicia faba* and *Z. mays*, *Ecballium cicutarium*, the fruits of apple and *Crataegus*, and the flowers of *Sambucus nigra* and *Arum italicum*.[45] Alanine and acetaldehyde are the proposed precursors of ethylamine in plant tissues.[45−47] Alanine is closer than acetaldehyde to L-theanine in the biosynthetic pathway.[48] Deng et al. reported the incorporation of radioactivity from [U−^{14}C]-alanine into theanine.[34] The formation of ethylamine by enzymatic decarboxylation of alanine has not been demonstrated previously. Since alanine is present in a wide variety of plant species (Table 1), it would be interesting to investigate if the enzyme catalyzing the conversion of alanine to ethylamine specifically accumulates in *C. sinensis* and whether this enzyme is crucial for the differential accumulation of ethylamine between *C. sinensis* and other plants. Ongoing research in our laboratory is aimed at isolating, purifying, and characterizing this enzyme from *C. sinensis*.

We detected differential accumulation of L-theanine between *C. sinensis* and other plant species (*C. nitidissima*, *C. japonica*, *Z. mays*, *A. thaliana*, and *S. lycopersicum*). We used a stable isotope labeling approach to elucidate the biosynthesis of L-theanine in *C. sinensis* and other plant species and measured immediate metabolites by mass spectrometry. *Arabidopsis* GSs were able to produce Gln and L-theanine, which suggested that GSs in a wide variety of plant species can also function in L-theanine synthesis. Ethylamine is crucial for the accumulation of L-theanine in plants. These results help to explain why *C. sinensis* produces specialized metabolites that determine the quality of tea and why *C. sinensis* is the most suitable material for tea production.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02437.

Primers of *AtGSs* used; analysis of ethylamine of soils used for selected plants by GC−MS (PDF)

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Notes

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■ ABBREVIATIONS USED

Ala, alamine; At, *A. thaliana*; Cj, *Camellia japonica*; Cn, *Camellia nitidissima*; Cs, *Camellia sinensis*; ESI, electrospray ionization; GC−MS, gas chromatography−mass spectrometer; Glu, glutamic acid; Gln, glutamine; GS, glutamine synthetase; IBCF, isobutyl chloroformate; IPTG, isopropyl-*β*-D-thiogalactopyranoside; IS, internal standard; LB, Luria−Bertani; MSTFA, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide; OPA, o-phthaladehyde; SDS, sodium dodecyl sulfate; SIM, selective ion monitoring; Sl, *S. lycopersicum*; TMEMD, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; TS, theanine synthetase; UPLC−QTOF-MS, ultraperformance liquid chromatography−quadrupole time-of-flight mass spectrometry; Zm, *Zea mays*

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