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Biosynthesis of theanine (γ-ethylamino-L-glutamic acid) in seedlings of *Camellia sinensis*

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Abstract

Theanine (γ-glutamylethylamide) is the most abundant free amino acid in tea seedlings, and is distributed in cotyledons, shoots and roots. Theanine was synthesised from ^{15}N-labelled $(NH_4)_2SO_4$, glutamic acid and alanine and from ^{14}C-labelled ethylamine in all parts of seedlings. When $(^{15}NH_4)_2SO_4$ was supplied to intact seedlings in liquid culture, incorporation of ^{15}N into theanine in roots was greater than in shoots. Incorporation into theanine was negligible in cotyledons, but theanine synthesis in roots and shoots was reduced in seedlings with cotyledons detached. Expression of theanine synthetase genes (*TS1* and *TS2*) was found in all organs, but the transcript level was significantly lower in cotyledons. These results suggest that theanine can be synthesised from glutamic acid and ethylamine derived from alanine in all parts of tea seedlings. However, supplied NH_3 exogenously to intact seedlings was converted to theanine mainly in roots. Amino acids stored in cotyledons may also be utilised for theanine synthesis in all parts of seedlings.

Keywords: *Camellia sinensis*; Theaceae; Tea; Biosynthesis; Theanine; γ-Ethylamino-L-glutamic acid; Amino acid

1. Introduction

Caffeine and theanine (γ-glutamyl-L-ethylamide) are the most abundant free nitrogenous compounds in tea (*Camellia sinensis*) leaves (Ashihara et al., 2008; Chu et al., 1997; Crozier et al., 2006). Theanine is a non-protein amino acid that was first discovered in tea leaves (Sakato, 1949). Theanine has been found in some *Camellia* plants and in a mushroom, *Xerocomus badius* (Casimir et al., 1960). Theanine has been extensively studied in relation to food science and human nutrition, because this compound has a unique taste characteristic of tea known as ''umami'' (Yamaguchi and Ninomiya, 2000). The effects of theanine on human health have recently been investigated (Chu et al., 1997; Juneja et al., 1999; Kakuda et al., 2000; Egashira et al., 2004). Theanine is known to act as an antagonist against paralysis induced by caffeine, and is also known to be a neurotransmitter in the brain; a relaxation-inducing effect in humans has been proposed (Chu et al., 1997).

Studies of theanine biosynthesis in tea plants were first carried out in the 1960s (Sasaoka et al., 1962, 1963; Konishi and Takahashi, 1969), since then, however, only a few studies have been published (Takeo, 1974; Konishi et al., 1978; Chu et al., 1997) compared with biomedical research. In the present paper we examined the biosynthesis of theanine in different parts of tea seedlings, using ^{15}N- and ^{14}C-precursors. Transcript levels of theanine synthetase genes (*TS1* and *TS2*) were also examined. The results obtained will provide the basis for further more detailed studies of theanine biosynthesis in plants.

2. Results and discussion

The concentrations of major amino acids in shoots, roots and cotyledons of 3-week-old tea seedlings are shown in Table 1. In all organs, theanine concentration was at least 10-fold higher than the next abundant amino acid, glutamine. Theanine concentration was in the order as roots > shoots > cotyledons. Other major amino acids were glutamine, glutamic acid, alanine, aspartic acid and asparagine. The glutamine level was higher than that of glutamic acid in shoots and roots, but the reverse was the case in cotyledons, where levels of alanine and aspartic acid were higher than glutamine.

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Table 1
Concentration of major amino acids (μmol g^{-1} fresh weight) in shoots, roots and cotyledons of 3-week-old tea seedlings

Organ	Glutamine	Asparagine	Glutamic acid	Aspartic acid	Theanine	Alanine
Shoot	5.2 ± 1.9	0.28 ± 0.05	2.5 ± 0.6	1.8 ± 0.2	63.9 ± 4.6	1.1 ± 0.1
Root	5.1 ± 0.5	0.42 ± 0.24	3.6 ± 0.7	1.9 ± 0.1	108.9 ± 11.2	4.5 ± 0.7
Cotyledon	3.7 ± 1.0	1.5 ± 0.82	7.2 ± 1.0	4.4 ± 0.2	23.1 ± 6.3	8.4 ± 2.6

Fresh weights of the shoot, root and pair of cotyledons were 24.9 ± 4.5, 135.5 ± 3.3 and 1288 ± 227 mg, respectively.

Theanine is synthesised from glutamic acid and ethylamine by theanine synthetase (EC 6.3.1.6) (Sasaoka et al., 1965). Ethylamine appears to be produced from alanine in tea plants by alanine decarboxylase (Takeo, 1974). The nitrogen in these organic compounds is derived from NH_4^+ or NO_3^-. It has been suggested that tea plants prefer ammonium ions as an inorganic nitrogen source for growth (Morita et al., 1998; Ruan et al., 2007); we therefore chose [^{15}N]ammonium sulphate, and the synthesis of theanine from this compound was also examined. As in other plants, NH_3 may be assimilated into glutamic acid via glutamine synthetase (GS) and glutamine: 2-oxoglutarate aminotransferase (GOGAT). Alanine may be synthesized from glutamic acid by alanine aminotransferase (Lea and Ireland, 1999).

To investigate the biosynthetic activity of theanine in cotyledons, shoots and roots, we examined the incorporation of ^{15}N from [^{15}N]ammonium sulphate, [^{15}N]glutamic acid, [^{15}N]alanine and from [^{14}C]ethylamine into theanine, using tissue segments of tea seedlings (Figs. 1 and 2). The rate of incorporation (expressed as ^{15}N atom % abundance) indicated that all organs of tea seedlings are able to synthesise theanine from all these compounds. Fig. 3 shows possible metabolic routes. In addition to [^{15}N]glutamic acid, the direct substrate of theanine synthesis, [^{15}N]alanine, was also efficiently incorporated into theanine (Fig. 1). It follows that [^{15}N]alanine was converted to ethylamine and used for theanine synthesis. Almost all of the radioactivity from [^{14}C]ethylamine taken up by the segments was converted to theanine (Fig. 2).

Using semi-quantitative reverse transcription-PCR, we studied the expression of two genes encoding theanine

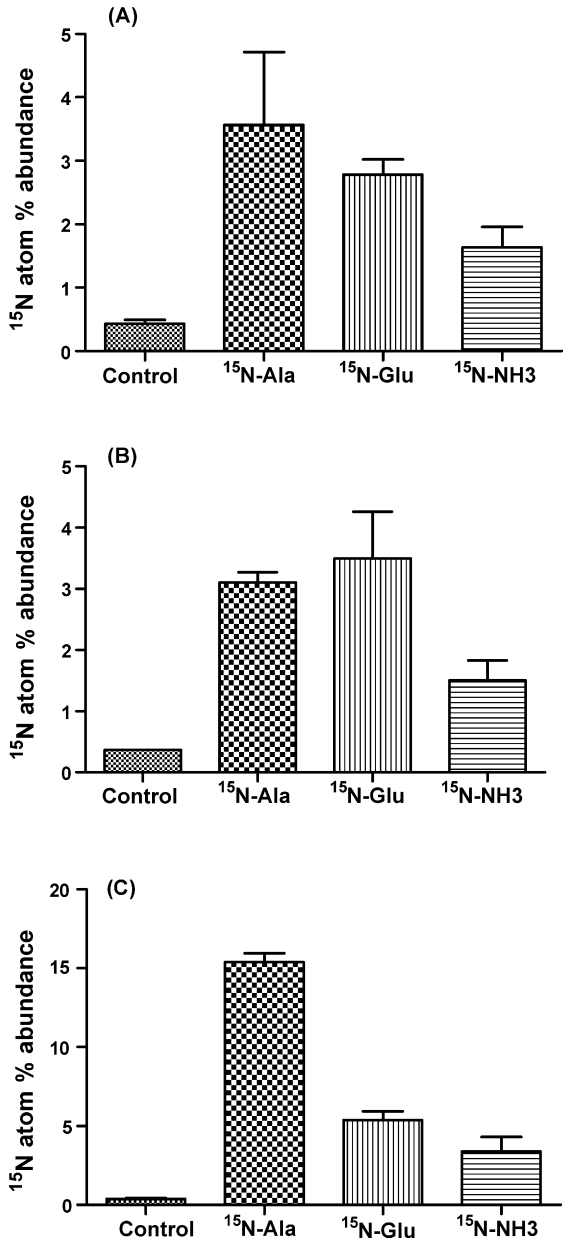

Fig. 1. Incorporation of ^{15}N from [^{15}N]alanine, [^{15}N]glutamic acid and [^{15}N]ammonium sulphate into theanine in segments of shoots (A), roots (B) and cotyledons (C) from 3-week-old tea seedlings. Rates of incorporation are expressed as ^{15}N atom % abundance. The natural ^{15}N abundance is also shown for comparison.

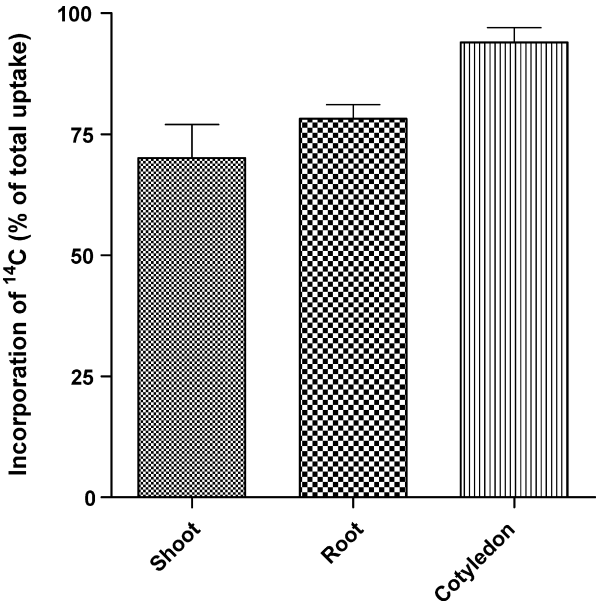

Fig. 2. Incorporation of ^{14}C from [^{14}C]ethylamine into theanine in the segments of shoots, roots and cotyledons from 3-week-old tea seedlings. Rates of incorporation are expressed as % of radiolabel taken up by the segments.

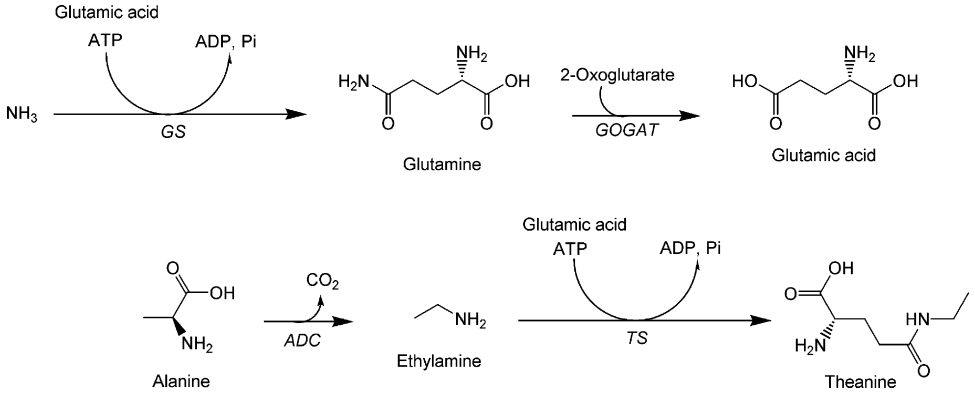

Fig. 3. Possible routes of theanine biosynthesis from NH_3, glutamic acid, alanine and ethylamine in tea seedlings.

synthetase (*TS1* and *TS2*) and, as constitutive control, α-tubulin (*Tua1*). Our study examined shoots, roots and cotyledons of 3-week-old tea seedlings, and mature leaves from 3-month-old young tea seedlings (Fig. 4). The amounts of transcripts of *TS1* were higher in shoots, roots and mature leaves than in cotyledons. Expression of *TS2* was greater in shoots than in other parts. Little difference was found in the amounts of transcripts of *Tua1* between the three components of tea seedlings and mature leaves. *TS1* was expressed equally in shoots and roots, but expression of *TS2* in roots was low.

The reaction mechanism of theanine synthesis (ADP-forming ligase reaction) is similar to that of glutamine synthetase. The nucleotide sequences of TS genes are very similar to those of tea glutamine synthetase (GS) genes. *TS1* has an overall 99% homology to *GS3* (GenBank accession no. AB117934), and *TS2* has 97% homology to *GS1* (GenBank accession no. AB115183). The homology between *TS1* and *TS2* was 83%. Detailed studies of proteins coding *TS1* and *TS2*, such as substrate specificity and cellular localization, are necessary to confirm the contribution of these isoforms of theanine synthetase to theanine synthesis in tea seedlings.

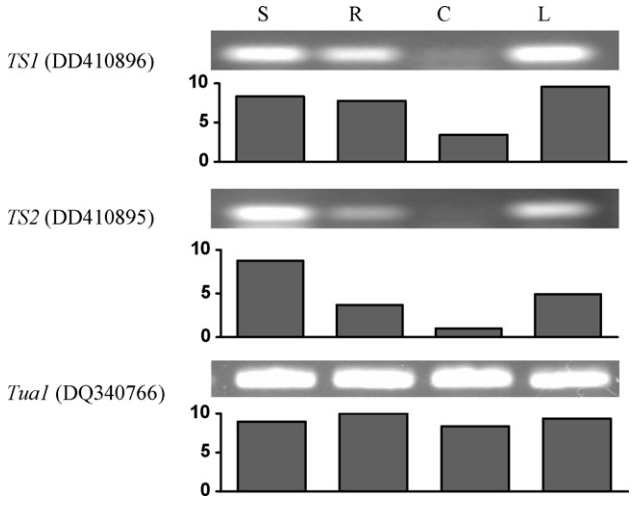

Fig. 4. Expression of *TS1*, *TS2* and *Tua1* in roots (R), shoots (S) and cotyledons (C) of 3-week-old tea seedlings. Transcripts of mature leaves (L) obtained from 3-month-old tea seedlings are also shown for reference.

In intact tea seedlings, nitrogen for theanine synthesis may be supplied from soil or from storage amino acids in cotyledons. To find the routes of theanine biosynthesis in intact tea seedlings, three-week-old seedlings were incubated with [^{15}N]ammonium sulphate for 3 days, and the incorporation of ^{15}N into theanine in roots, shoots and cotyledons was examined (Fig. 5). When whole seedlings were used, incorporation of ^{15}N into theanine in roots increased essentially linearly with incubation time. ^{15}N was also incorporated into theanine in shoots, but at a lower rate than in roots. Incorporation into cotyledons was negligible (Fig. 5A).

Rates of incorporation of ^{15}N from [^{15}N]ammonium sulphate into theanine in roots and shoots were reduced significantly when cotyledons were removed from the seedlings (Fig. 5B). This is probably due to the reduction in energy or carbon supply for amino acid synthesis in such seedlings.

The present results suggest that all parts of tea seedlings are able to synthesise theanine from glutamic acid and ethylamine. Ammonium ions absorbed exogenously by roots are utilised mainly for theanine in roots; smaller amounts are transported to shoots and used for theanine synthesis. Theanine produced in roots may also be transported to shoots. The pattern of expression of the two theanine synthetase genes registered in GenBank differs in roots and shoots, although expression of these genes is low in cotyledons. Since the nucleotide sequences of genes of theanine synthetase are very similar to those of glutamine synthetase, it is unclear whether theanine is synthesised by specific theanine synthetase, by glutamine synthetase, or by other enzymes such as glutaminase or γ-glutamyltranspeptidase, which are found in bacteria (Tachiki et al., 1998; Suzuki et al., 2002).

3. Experimental

3.1. Plant materials

Seeds of tea (*Camellia sinensis*) were collected from the tea plantation at the National Research Institute of Vegetables, Ornamental Plants and Tea, Makurazaki, Japan. To promote germination, tea seeds were sterilised with sodium hypochlorite and the seed coats were removed. They were then cultured on

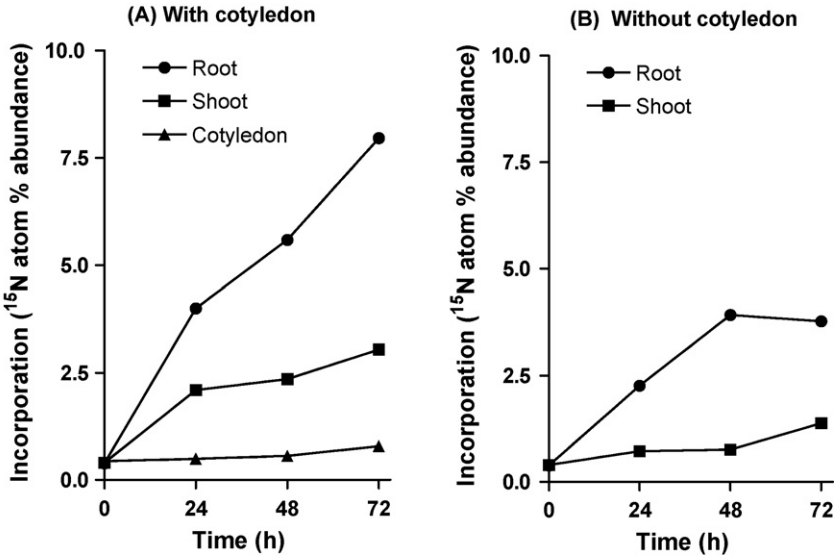

Fig. 5. Time course of [^{15}N]theanine formation from [^{15}N]ammonium sulphate in intact 3-week-old tea seedlings. Rates of incorporation are expressed as ^{15}N atom % abundance. The natural ^{15}N abundance is shown at 0 h. The values are the averages of two repetitions.

0.55% agar gel with no additional compounds in natural light at 25 °C.

3.2. Stable isotopes and radioisotopes

[^{15}N]Ammonium sulphate, [^{15}N]L-glutamic acid and [^{15}N]L-alanine were purchased from ISOTEC Biochemicals, Miamisburg, OH, USA (all isotopes contain more than 98 atom% ^{15}N). [1-^{14}C]Ethylamine hydrochloride (specific activity 2.22 GBq mmol^{-1}) was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO, USA.

3.3. Determination of endogenous amino acid content

Plant materials were homogenized in a pestle and mortar with 80% (v/v) aqueous MeOH. The MeOH-soluble fraction was evaporated in vacuo, and the pellet obtained was dissolved in a small amount of distilled water. After brief centrifuging at 12,000 × *g*, the supernatant was freeze dried, and was used for amino acid analysis. Amino acids, including theanine, were separated and analysed using a HPLC system with a fluorescent detector adopted for free amino acid analysis (Kotaniguchi et al., 1987).

3.4. Administration and analysis of ^{15}N-labelled compounds

The ^{15}N-tracer experiments were carried out as specified by Ito and Ashihara (1999). The segments of roots (ca. 150 mg), shoots (ca. 40 mg) and cotyledons (ca. 500 mg) obtained from 3-week-old tea seedlings were placed in flasks with 2 ml of half-strength Murashige-Skoog (1/2 MS) salt medium (Murashige and Skoog, 1962) containing ^{15}N-labelled compounds (4 mg). The flasks were incubated in an oscillating water bath at 25 °C for 24 h. For the experiments using whole seedlings, 3-week-old seedlings were incubated in liquid 1/2 MS medium containing ($^{15}NH_4)_2SO_4$ (2 mg ml^{-1}) in a culture room with a light (18 h)/dark (6 h) cycle at a light intensity of 150 μmol^{-2} s^{-1} at 25 °C. Samples were collected after 24, 48 and 72 h of incubation, and the incorporation of ^{15}N into theanine in shoots, roots and cotyledons was determined.

For analysis of ^{15}N-theanine, samples were homogenized using a mortar and pestle with 80% aqueous MeOH. The resulting MeOH-soluble fraction was concentrated and was applied to microcrystalline cellulose TLC plates (Merck, Darmstadt, Germany). The solvent system, *n*-BuOH/HOAc/H_2O (4:1:2), was used for separation of theanine from other ninhydrin-positive compounds. Spots of theanine were scraped off and eluted from the cellulose support with 80% aqueous MeOH. ^{15}N-Theanine was eluted from cellulose powders, and the relative isotopic abundance of ^{15}N was analysed by EA-IRMS (elemental analyser combustion isotope ratio mass spectrometry) using a ANCA-SL device (Sercon Limited, Crewe, Cheshire, UK). To avoid the saturation of Faraday collectors, samples with high ^{15}N/^{14}N ratio were analysed using reduce sample sizes up to 25 μg N. The linearity of ANCA-SL instrument has a standard deviation less than 0.7‰ and the range is from 20 to 200 μg N when using standard material of natural abundance.

3.5. Administration and analysis of [^{14}C]ethylamine

The ^{14}C-tracer experiments were performed according to our earlier report (Ashihara et al., 1997). Segments of shoots, roots, cotyledons (ca. 100 mg fr. wt) and 2.0 ml of 30 mM K-Pi buffer (pH 5.6) containing 20 mM sucrose and 1% Na-ascorbate were placed in the main compartment of a 30-ml Erlenmeyer flask. The flask was fitted with a small glass tube that contained a piece of filter paper impregnated with 0.1 ml of 20% KOH in the centre well, to collect $^{14}CO_2$. Each reaction

was begun by adding the solution of radioactive compounds to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 27 °C for 18 h.

For ^{14}C-analysis, samples were homogenized with 80% aqueous MeOH using a pestle and mortar. The resulting methanol-soluble fraction was concentrated and separated by TLC, using cellulose plates and the solvent systems, PhOH/H_2O (3:1). Radioactivity on the TLC sheet was determined using a Bio-Imaging Analyser (Type, FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan).

3.6. Determination of transcript amounts of TS

Total RNA was extracted from different parts of the tea seedlings, and was purified using the method reported previously (Li et al., 2008). DNA-free total RNA was employed for first strand cDNA synthesis, using a RNA PCR core kit (Applied Biosystems, Roche Molecular systems, USA). The PCR reaction mixture (25 μl) contained 40 ng cDNA and 12.5 μl GoTaq Green mastermix (Invitrogen, Carlsbad, CA, USA); 28–30 cycles were carried out with a program of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s. The reaction product was visualized by UV light on 2% agarose gels stained with ethidium bromide, using a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan). The amounts of the resulting products were determined densitometrically after gel electrophoresis, to find the relative level of transcripts of each gene. The gene specific primers were prepared from the tea sequences registered in the GenBank. The sequences of primers used in this work were as follows:

TS1 (Theanine synthetase, GenBank accession no. DD410896), 5′-AATTCCAAGTTGGGCCTTCT-3′ and 5′-GCTTCAATCCAAGCTTTTCG-3′
TS2 (Theanine synthetase, GenBank accession no. DD410895), 5′-CTCATCAACCTCAACCTTTC-3′ and 5′-TGCTTGAGGGTAGATAATGA-3′
Tua1 (α-tubulin 1, GenBank accession no. DQ340766), 5′-TGGGTTCAAGTGTGGAATCA-3′and 5′-TCCATACCCT-CCCCAACATA-3′

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