Ethylamine Content and Theanine Biosynthesis in Different Organs of *Camellia sinensis* Seedlings

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We examined the distribution of ethylamine, glutamic acid and alanine, which are utilized in theanine biosynthesis, and other major amino acids in leaves, stems, cotyledons and roots of 6-week-old tea seedlings. Ethylamine and glutamic acid, which are substrates of theanine synthetase, were distributed almost uniformly in all parts of the seedlings; the contents in μmol/g fresh wt varied from 0.44–0.88 (ethylamine) and 1.6–2.4 (glutamic acid). The content of alanine, a possible precursor of ethylamine synthesis, was significantly higher in roots (3.1 μmol/g fresh wt) than in other parts. Incorporation of radioactivity from [U-^{14}C]-alanine into theanine was also higher in roots than in other organs. In 10-week-old seedlings, [1-^{14}C]ethylamine was converted to theanine in young and developed leaves, stems, main and lateral roots; the highest rates of conversion were detected in the main and lateral roots. These results suggest that the theanine synthesis preferentially takes place in roots but is not restricted to them; substrates and the enzymatic machinery for theanine synthesis are available in all parts of tea seedlings.

Key words: Alanine, Ethylamine, Theanine Biosynthesis, *Camellia sinensis*

Introduction

Theanine (γ-glutamyl-L-ethylamide) is the most abundant free amino acid in tea leaves (Chu *et al.*, 1997; Crozier *et al.*, 2006). Theanine was first discovered in tea leaves by Sakato (1949) and has since been found in some *Camellia* plants (Nagata and Sakai, 1984) and in a mushroom, *Xerocomus badius* (Casimir *et al.*, 1960). Although there have recently been extensive studies on theanine in relation to human nutrition and health, few reports exist from the viewpoint of plant physiology, *i.e.*, biosynthesis, metabolism and its role *in planta*.

It has been suggested that theanine is synthesized from glutamic acid and ethylamine by theanine synthetase (L-glutamate-ethylamine ligase, EC 6.3.1.6) in roots of tea plants, and is transported to shoots (Sasaoka *et al.*, 1965). In our previous report (Deng *et al.*, 2008), we used 3-week-old seedlings and demonstrated that theanine is the most abundant free amino acid in cotyledons, shoots and roots; we suggested that theanine is synthesized in all parts of the seedlings. Distinct expression of *TS*, reported as genes encoding theanine synthetase (GenBank accession numbers: DD410896 and DD410895), was found in roots and shoots, but the expression was low in cotyledons.

In the present follow-up, we examined the distribution of ethylamine in different parts of tea seedlings. Ethylamine appears to be produced from alanine in tea plants by alanine decarboxylase (Takeo, 1974, 1978), and is used for theanine synthesis. Tsushida and Takeo (1984) reported the presence of ethylamine in fresh tea shoots, and suggested that it is produced as a degradation product of theanine which had been transported from the roots. The distribution of ethylamine in different organs has not yet been examined, however.

We further compared the incorporation rate of radioactivity from ^{14}C-labelled alanine and ethylamine into theanine in roots, leaves, and stems of 6- and 10-week-old tea seedlings. We found that all parts of tea seedlings are able to synthesize theanine, although the synthetic activity estimated from the incorporation of labelled alanine and ethylamine was higher in roots than in leaves.

Material and Methods

Seeds of tea (*Camellia sinensis*, cv. Shuchazao) were obtained from the Botanical Garden of Anhui Agricultural University, Hefei, China. We used seedlings grown for 6 weeks on 0.55% agar gel without nutrients at 25 ºC in natural light. The ^{14}C-ethylamine experiments used 10-week-old seedlings. In those experiments, the 6-week-old seedlings referred to above were transferred to the liquid culture medium described by Konishi *et al.* (1978).

Ethylamine and amino acids were extracted and analyzed according to Tsushida and Takeo (1984), with a slight modification as follows. Segments of roots, cotyledons, leaves and stems were immediately frozen in liquid N_2 and freeze-dried overnight. The powdered dry samples were dissolved in 5 ml of distilled water and heated for 5 min at 80 ºC. The homogenates were left at room temperature for 2 h and filtered. The resulting samples were adjusted to pH 8.0 with 50 mM borate buffer and used for HPLC analysis with a fluorescent detector adapted for free amino acid analysis (Kotaniguchi *et al.*, 1987).

The ^{14}C-tracer experiments were performed using [U-^{14}C]alanine (specific activity 4.7 GBq/mmol; Moravek Biochemicals, Brea, CA, USA) and [1-^{14}C]ethylamine hydrochloride (specific activity 2.2 GBq/mmol; American Radiolabeled Chemicals, St. Louis, MO, USA). Portions of the samples (*ca.* 100 mg fresh wt) were mixed in the main compartment of a 30-ml Erlenmeyer flask with 2.0 ml of 30 mM potassium phosphate buffer (pH 5.6) containing 20 mM sucrose and 1% Na-ascorbate. The flask was fitted with a small glass tube containing 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 (37 kBq, 10 μl) to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 27 ºC for 18 h. For analysis of ^{14}C-metabolites the samples were homogenized with 80% aqueous methanol using a pestle and mortar. The resulting methanol-soluble fraction was concentrated and separated by TLC using cellulose plates (Merck, Darmstadt, Germany) and the solvent systems phenol/water (3:1, v/v) and *n*-butanol/acetic acid/water (4:1:2, v/v/v). Radioactivity on the TLC sheet and in the liquid fraction was determined using a bio-imaging analyser (Type FLA-2000, Fuji Photo Film, Tokyo, Japan) and a multi-purpose scintillation counter (Type LS 6500, Beckman, Fullerton, CA, USA) with scintillation fluid (Amersham ACS-II, GE Healthcare, Tokyo, Japan).

Results and Discussion

Ethylamine appears to be a unique metabolite in tea plants, and is used for the synthesis of theanine (Sasaoka *et al.*, 1965; Deng *et al.*, 2008). Tsushida and Takeo (1984) reported the ethylamine content in fresh tea shoots, but no information is available on the distribution of ethylamine in other organs of tea plants. In the present study, we examined the distribution of ethylamine and major free amino acids, including theanine, in different parts of 6-week-old seedlings. Tea seedlings in this growth stage were chosen because differentiation of leaves and roots is completed and no external nutrients are necessary for growth until this stage has been reached. All the nitrogen atoms in theanine and ethylamine are derived from storage materials in cotyledons.

Table I shows the content of major free amino acids in different parts of 6-week-old tea seedlings. The theanine content was always higher than that of other amino acids in all parts of the

Table I. Content [average values expressed as μmol/g fresh wt ± SD (n = 3)] of ethylamine and main amino acids in different organs of 6-week-old tea seedlings.

Organ	Ethylamine	Ala	Theanine	Glu	Gln	Asp	Asn
Cotyledons	0.55 ± 035	2.26 ± 0.68	11.38 ± 0.81	1.79 ± 0.58	1.42 ± 0.57	1.39 ± 0.09	0.28 ± 0.05
Roots	0.66 ± 012	3.05 ± 0.16	39.00 ± 4.74	1.64 ± 0.32	1.56 ± 0.21	0.91 ± 0.13	0.20 ± 0.02
Leaves	0.88 ± 007	0.55 ± 0.06	14.69 ± 1.95	2.30 ± 0.36	1.00 ± 0.05	1.77 ± 0.26	0.44 ± 0.15
Stems	0.44 ± 002	0.95 ± 0.17	33.85 ± 3.68	2.39 ± 0.59	8.19 ± 1.14	1.64 ± 0.75	0.44 ± 0.02

Fresh weights (g) per organs are as follows: Cotyledons, (1.11 ± 0.18); roots, (1.05 ± 0.03); leaves, (0.52 ± 0.08); stems, (0.53 ± 0.02).

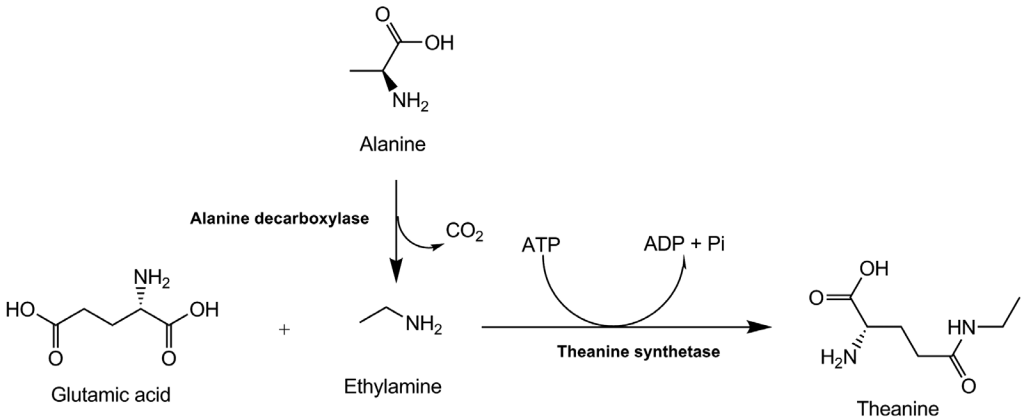

Fig. 1. Theanine synthesis from glutamic acid and ethylamine. Ethylamine formation from alanine is also shown.

seedlings. Ethylamine was found in all parts of the seedlings, but the greatest content was in leaves, at almost 1 μmol/g fresh wt. This is equivalent to approximately 4 μmol/g dry wt, slightly lower than the value in developed tea leaves (Tsushida and Takeo, 1984). The second highest free amino acid was alanine in roots and cotyledons, but its content in leaves and stems was lower than those of glutamic acid, glutamine and aspartic acid. The content of alanine in leaves was 5.6 times less than in roots. The theanine content is also high in roots, but the content of other amino acids in each organ was less variable, although the glutamine content was high in stems.

In tea seedlings the distribution of amino acids in different organs appears to depend strongly on the growth stage. Free amino acids and those derived from storage proteins in cotyledons decrease with age; in contrast, free and protein amino acids increase in developing parts. Transport of theanine from roots to shoots may also be involved in these changes. Our results nevertheless suggest that ethylamine is distributed in all parts of tea seedlings.

Table II shows the incorporation of [U-^{14}C]-alanine into theanine in roots, leaves and stems isolated from tea seedlings. Theanine biosynthesis from alanine was found to occur in each organ. The greatest activity was in roots. Although the possibility of conversion of alanine to glutamic acid for the synthesis of theanine cannot be excluded, most radioactivity appeared to be incorporated into theanine after it was converted to ethylamine (Fig. 1). Takeo (1974) reported that a significant amount of ^{14}C from [U-^{14}C]alanine was incorporated into the ethylamide of theanine in roots of 60-day-old tea seedlings.

Table III shows the incorporation of [1-^{14}C]-ethylamine into theanine, in young and developed leaves, stems, main and lateral roots of 10-week-old tea seedlings. Theanine biosynthesis was found in all parts of tea seedlings. The highest biosynthetic

Table II. Incorporation of radioactivity from [U-^{14}C]-alanine into theanine in different organs of 6-week-old tea seedlings.

Organ	Incorporation [pmol/100 mg fresh wt]
Leaves	15.42 ± 0.82
Stems	8.40 ± 0.23
Roots	86.70 ± 3.23

Average values ± SD (n = 3) are shown.

Table III. Incorporation of radioactivity from [1-^{14}C]-ethylamine hydrochloride into theanine in different organs of 10-week-old tea seedlings.

Organ	Incorporation [nmol/100 mg fresh wt]
Young leaves	0.23 ± 0.01
Developed leaves	1.34 ± 0.08
Stems	0.64 ± 0.01
Main roots	2.45 ± 0.45
Lateral roots	2.48 ± 0.64

Average values ± SD (n = 3) are shown

activity was in main and lateral roots. Preliminary time course studies showed that incorporation of radioactivity into theanine from [1-^{14}C]ethylamine in leaves increased gradually up to 24 h, then decreased slightly (12%) at 48 h (data not shown). Slow degradation of theanine might therefore take place in leaves. Although conversion of theanine to catechins has been reported in tea leaves (Kito *et al.*, 1968), we did not detect any radioactivity in catechins in leaves up to 48 h after administration of [^{14}C]ethylamine (data not shown).

Our results indicate that all parts of tea seedlings have the ability to synthesize theanine. Theanine synthesis is more rapid in roots. Substrates of theanine biosynthesis and possibly theanine synthetase appear to be distributed in all parts of tea seedlings. Our attempt to compare the activity of theanine synthetase in different organs was not successful, probably due to the instability of the enzyme.

One reason for theanine synthesis *in planta* might be detoxification of ammonia absorbed by roots. This compound is accumulated in all parts of seedlings, however, even in seedlings grown without any nutrition as in the present study. Theanine might act as an easily transported nitrogen compound and be converted to other nitrogenous compounds after degradation to glutamic acid and ethylamine. Further studies would be necessary to test this hypothesis.

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