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Short communication

Effect of salt treatment on theanine biosynthesis in *Camellia sinensis* seedlings

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ABSTRACT

Theanine synthetase (TS) is an enzyme involved in theanine biosynthesis in tea plants. Recent studies have revealed that theanine biosynthesis, derived from nitrogen metabolism in tea (*Camellia sinensis* L.) plants, could be influenced by salt treatment. We have characterized CsTS at the molecular and biochemical level. The expression pattern of CsTS protein was examined by western blot using a self-prepared polyclonal antibody with high specificity and sensitivity. The effect of salt treatment on the levels of theanine synthesis was investigated in this study. Levels of theanine and the total free amino acids were gradually increased in shoots, and reached the maximum on the 8th day after treatment (DAT). The immunoblotting analysis suggested the accumulation of CsTS protein had increased gently up to 8 DAT, and subsequently declined, both in roots and shoots, which is one of the main evidences that resulted in the variation of theanine concentration under salt treatment. Together, these data revealed that theanine synthesis takes place both in root and shoot and CsTS accumulation is positively affected by salt treatment.

1. Introduction

Tea plants are often influenced by periods of soil and atmospheric water deficits, or high soil salinity, during their life cycle. The primary metabolism in plant, including photosynthesis, together with cell growth, can be affected by water or salt stress [1]. Theanine (γ-glutamyl-L-ethylamide), an important secondary metabolite, is one of the most abundant free nitrogenous compounds and amino acids found in tea (*Camellia sinensis*) leaves [2–4]; and also distributed in all parts of young tea seedlings [5]. Theanine is synthesized from glutamic acid and ethylamine by theanine synthetase (L-glutamate-ethylamine ligase, EC 6.3.1.6) in all parts of tea seedlings, as emphasized [6]. However, the greatest biosynthetic activity was found in roots. One reason for theanine synthesis in plants might be detoxification of ammonia, absorbed by tea roots. Theanine might act as an easily transported nitrogenous compound after degradation to glutamic acid and ethylamine [6]. However, the absorption of ammonium would be affected by salt treatment, due to the previous report [7]. Consequently, theanine biosynthesis that is derived from nitrogen metabolism in tea plants could also be influenced by salt treatment. Nevertheless, theanine synthesis responding to salt treatment in tea plants remains unclear.

In this study, theanine synthetase was investigated at the protein level through the acquisition of a specific antibody in tea seedlings, both controlled and treated by NaCl. This experiment also investigated the trend in the concentration of main free amino acids, including theanine, along with the expression level of theanine synthetase in tea seedlings by salt treatment, within 22 DAT. Here, we demonstrated that the total concentration of major amino acids was increased either in roots or shoots. Therefore, the objective of this investigation was to evaluate the effect of salt treatment on the expression patterns of theanine synthetase protein and the concentration of theanine in tea plants, and at the same time try to correlate these effects with changes in theanine accumulation, with a view to a better understanding of the mechanism of salt treatment in theanine biosynthesis.

2. Materials and methods

2.1. Plant materials and stress treatment

Seeds of tea (*C. sinensis*) were collected from tea plantation at the Anhui Agricultural University, Hefei city, Anhui Province, China. To promote germination, tea seeds were sterilized with sodium hypochlorite and the seed coats were removed. Seeds were pregerminated on the filter papers and culture conditions, as previously described [8]. For preparation of experimental materials, half-

Abbreviations: Cs, *Camellia sinensis*; DAT, day after treatment; TS, theanine synthetase.

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strength Murashige–Skoog (1/2 MS) medium [9] supplemented with NaCl (150 mM) was watered to the seedlings everyday. Seedlings were collected at 1, 8 and 22 DAT and washed with distilled water. Samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.2. Construction of the pEasy-TS fusion protein expression vector

The coding region of theanine synthetase (TS) was amplified by RT-PCR from total RNA of *C. sinensis* young leaves using gene-specific primers: 5′-GCCAGATCTATGTCTCTTCTTTCCG-3′ (forward, *Bgl II* site underlined) and 5′-TTGTCGACTTACGGTTTCCAGAGG-3′ (reverse, *Sal I* site underlined). The PCR reaction was performed in 30 μL containing 1.6 μg cDNA from tea leaves as template using the LA Taq polymerase system (Takara, Otsu, Japan) by 30 cycles (denaturation for 60 s at 94 °C; annealing for 60 s at 56 °C; extension for 80 s at 72 °C) followed by a final elongation step for 10 min at 72 °C. Amplified PCR products were digested with the respective restriction enzymes and cloned in frame with pEasy-E1 vector (TransGen, Beijing, China), resulting in pEasy-E1-CsTS. After confirming the cloned fragments by DNA sequencing, pEasy-E1-CsTS was transformed into the Rosseta good (DE3) cells and the recombinant CsTS protein expressed according to the pEasy-E1 vector manufacturer's instructions (TransGen, Beijing, China).

2.3. Expression of the recombinant protein

Cells were grown at 37 °C in LB media containing ampicillin (50 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) overnight. Following centrifugation, *Escherichia coli* cells were adjusted to $OD_{600} = 0.5$, the production of recombinant protein was induced by adding IPTG to a final concentration of 1 mM.

2.4. Purification of the recombinant protein

The cells were harvested by centrifugation at 4 °C, and cell pellets were re-suspended in binding buffer containing 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl (pH 8.0). After cells were disrupted on ice by ultrasonication, the pellets were re-suspended in a buffer with 8 M urea, 100 mM Na_2HPO_4, 100 mM Tris–HCl (pH 8.0) until it changed to a transparent solution. After centrifugation at 12,000×*g* for 20 min at 4 °C, the cleared supernatant was applied to a Ni NTA His Bind resin column (Qiagen, Shanghai, China) to bind the recombinant protein. The column was washed twice with washing buffer [8 M urea, 100 mM NaH_2PO_4, 100 mM Tris–HCl (pH 8.0)], and the bound recombinant protein was eluted with elution buffer [8 M Urea, 50 mM NaH_2PO_4, 300 mM NaCl (pH 8.0)]. The purified recombinant TS protein was immediately dialyzed against the buffer [50 mM Tris, 30 mM KCl, 1 mM DTT, 20% (v/v) glycerol (pH 7.9)].

2.5. Antibody production and purification

The purified recombinant protein was used to raise antibodies in white rabbits. Primary immunization was performed using 0.25–0.50 mg of recombinant protein in Freund's complete adjuvant. After 1 month, the rabbit was boosted three times with 0.25–0.50 mg of recombinant protein to complete Freund's adjuvant at 2-week intervals, and the antiserum was collected 7 days after the final injection. Purification of rabbit IgG was performed according to methods described by Tian et al. [10].

2.6. SDS-polyacrylamide gel electrophoresis and western analysis

Western blot, ELISA and protein dot blot analysis were performed as described by Tian et al. [10]. The method for extraction of total soluble protein is similar to a previous report [11], but modified as follows. Samples (about 200 mg) were ground in liquid nitrogen. An extraction buffer [50 mM Tris (pH 7.5), 20 mM KCl, 13 mM DTT], was added in a 1:5 ratio (plant tissue: buffer). After homogenization, the sample was re-extracted using 20 μL phenylmethanesulfonyl fluoride (PMSF) and 40 μL nonylpheno xypoly (ethyleneoxy) ethanol (NP-40). The supernatant was collected and precipitated with 3–5 volumes of 10% (w/v)

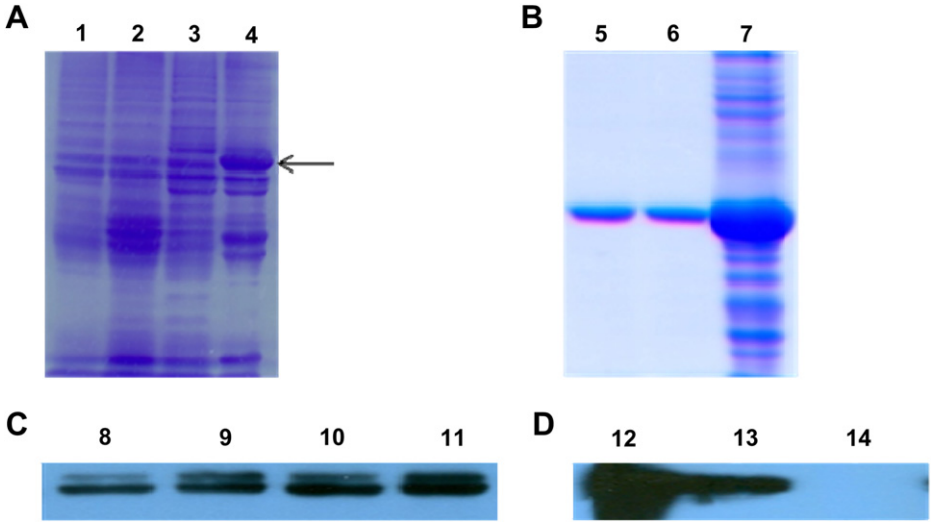

Fig. 1. Expression, purification of recombinant TS protein and characterization of the TS antibodies. (A) Expression of the recombinant protein at 37 °C for 6 h. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, the supernatant of control; lane 2, the supernatant of induction; lane 3, the precipitate of control; lane 4, the precipitate of induction. Arrow indicated the target recombinant protein. (B) The expressed His-tag fusion protein was purified by affinity chromatography. The precipitate from induced cultures was loaded on the column. After purification, proteins were separated by SDS-PAGE and stained with Coomassie Blue. Lanes 5, 6, the purified target protein; lane 7, non-purified proteins. (C) Protein western blot of 1:2000 dilution of the antibody reacting with gradient amount of total proteins from tea leaves. 10, 15, 20, 25 μg total protein of tea leaves were loaded to lanes 8–11, respectively. (D) The specificity of antibodies by western blot analysis. Lane 12, the TS antibody; lane 13, the purified target protein; lane 14, the precipitate of control, not induced by IPTG.

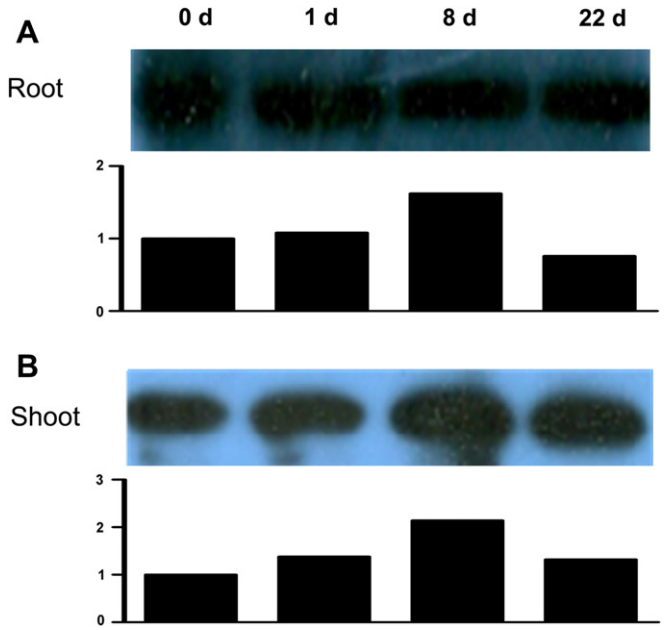

Fig. 2. Western blot analysis of TS expression in roots (A) and shoots (B) of tea seedlings during salt stress at day 0, 1, 8 and 22.

trichloroacetic acid in cold (−20 °C) acetone for 2–4 h. After centrifuge, the precipitate was washed by 0.07% DTT (w/v) in cold (−20 °C) 80% acetone. Proteins were dried under vacuum, stored at −20 °C or re-suspend in a rehydration buffer {7 M urea, 2 M thiourea, 0.4% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane (CHAPS), 60 mM DTT, 0.4% (w/v) PMSF} and stood at room temperature for 2 h. After the centrifuge, the supernatant was collected and stored at −80 °C until use. The total amount of protein was measured with a Bradford protein assay kit (Bio-Rad laboratories) using bovine serum albumin as standard. Proteins were separated by SDS-PAGE (10% resolving gel, 4% stock gel) and transferred to polyvinylidene fluoride membranes using a semidry electroblotter. After blocking 1 h in a TBS buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl] with 5% (w/v) non-fat dried milk at room temperature, membranes were incubated with Rat-anti-TS antibody for 1 h. After being washed three times in TBS buffer, membranes were incubated with anti-Rat (IgG) (Sigma, St. Louis, MO, USA) as secondary antibody and complexes were visualized using a detection kit (Thermo, Rockford, USA).

2.7. Exaction and determination of endogenous amino acids

Amino acids were extracted and analyzed according to Tsushida and Takeo [12], with a slightly modification as follows. Roots and shoots were immediately moved into an oven at 103 ± 2 °C until completely dried. The powdered dry samples (0.5 g) were dissolved in 95 mL of distilled boiling water and heated for 45 min at 100 °C. The homogenates were stood at room temperature and adjusted to 100 mL with distilled water. Samples were adjusted to a pH value of 8.0 with 50 mM borate buffer, before amino acid analysis. Amino acids, including theanine, were separated and analyzed using an HPLC system with a fluorescent detector adopted for free amino acid analysis [13]. Standards of amino acids were obtained from Sigma (St. Louis, MO, USA).

3. Results and discussion

3.1. Purification of recombinant CsTS protein and characterization of CsTS antibody

The appropriate experimental condition for the recombinant protein expression was determined under different temperatures (16–37 °C) and induction time (1–12 h) (data not shown) with 1 mM IPTG. Increased expression levels of the recombinant protein were detected under a 6 h induction at 37 °C more than other conditions; therefore we used the condition of 6 h induction at 37 °C for recombinant protein (Fig. 1A, lane 4). The recombinant protein was purified via the His-tag by affinity chromatography (Fig. 1B, lanes 1, 2). Polyclonal antibodies against TS, obtained from rabbits, are still highly sensitive when they were diluted at 1:2000. The sensitivity was tested by a western blot using gradient amount of total proteins from tea leaves (Fig. 1C). Also the specificity of the antibody preparation was performed with a western blot using the purified TS (Fig. 1D, lane 2).

3.2. Effect of salt treatment on seedling growth

The treatment of tea plants tested under salt environment, showed an ability to withstand a moderate salt concentration in

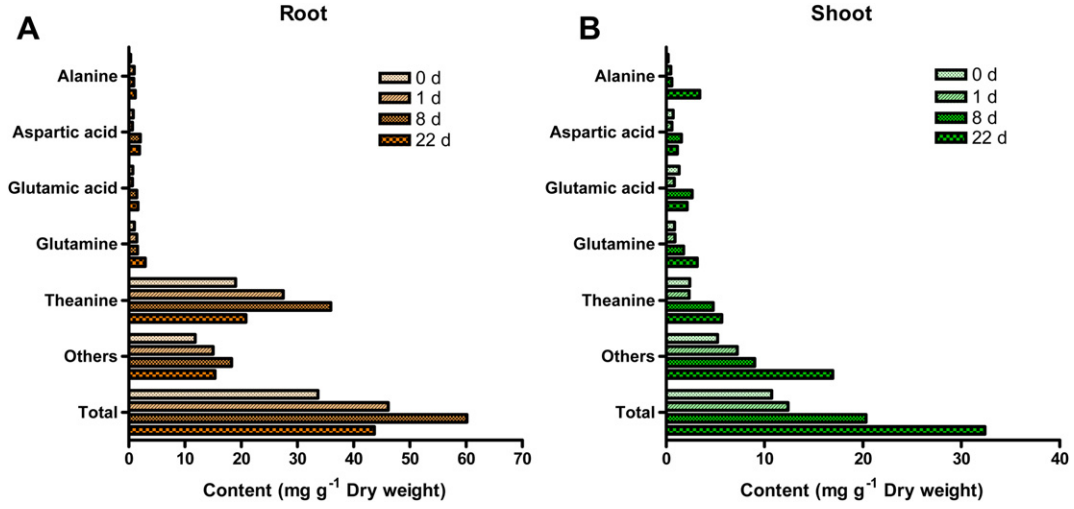

Fig. 3. Concentrations of theanine and other main amino acids in roots (A) and shoots (B) of tea seedlings during salt stress at day 0, 1, 8 and 22.

150 mM. During this salt treatment period (ca. 3 weeks), tea plants were shown a salt-tolerant property until 8 DAT, then shown a salt-sensitive phenomenon (wilting). Our preliminary experiments indicated that there is no significant difference between control plants and plants treated by nutrient solution containing 100 mM of NaCl; however, the tea plant growth was greatly restrained in a 200 mM concentration of NaCl.

3.3. Expression of TS during salt treatment

We studied the CsTS expression using immunoblotting analysis. The protein accumulation of CsTS varied in a similar pattern between roots and shoots during salt treatment, as we examined young tea seedlings. The CsTS protein increased gradually after treatment and reached the maximum at 8 DAT, and then decreased (Fig. 2 A and B). The accumulation of TS protein basically coincided with the concentration changes of theanine during salt treatment (Fig. 3).

3.4. Concentrations of theanine and main amino acids

In total, 17 free amino acid contents from the roots and shoots were determined in this study, both control and NaCl treated (1, 8 and 22 days) tea seedlings. The concentration of typical amino acids and the total amino acids are illustrated in Fig. 3. The average content of total free amino acids at 0, 1, 8 and 22 DAT was, respectively, 33.65, 46.14, 60.13, 43.68 mg g^{-1} dry weight in roots and 10.72, 12.37, 20.30, 32.41 mg g^{-1} dry weight in shoots, showing that total free amino acid contents were significantly increased by salt treatment. The maximum content was observed at 8 DAT in roots, while it was still increasing at that time from shoots. Compared to shoots, the concentration of total free amino acids was much higher in roots, which nevertheless, was almost 3 times higher than 22 DAT. Parida et al. [14] reported that the NaCl treatments on *Bruguiera parviflora* at high salt concentration showed an increase in the total amino acid pool by decreasing protein

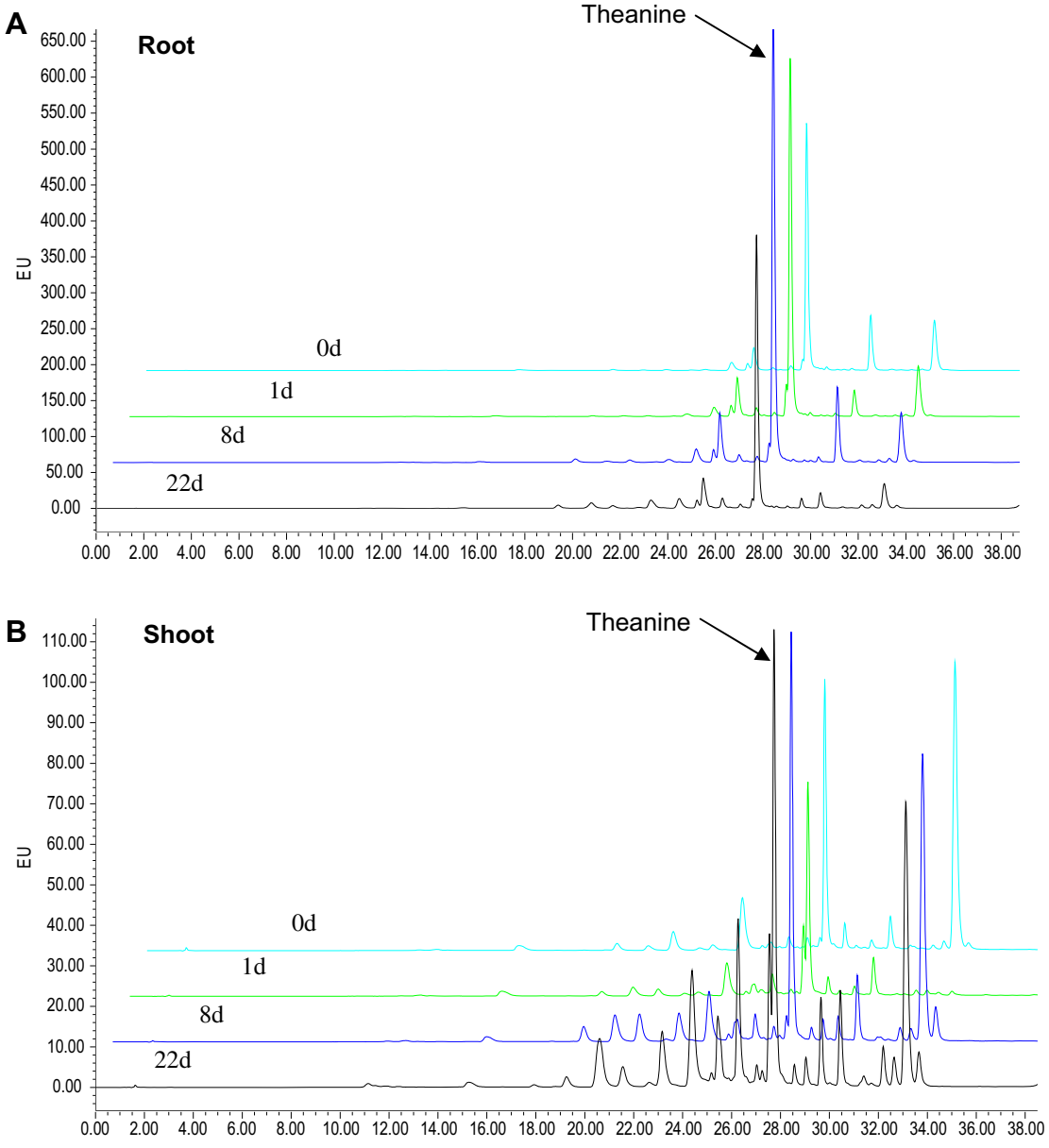

Fig. 4. HPLC-chromatograms for theanine identification in roots (A) and shoots (B) of tea seedlings by salt stress for 0, 1, 8 and 22 days.

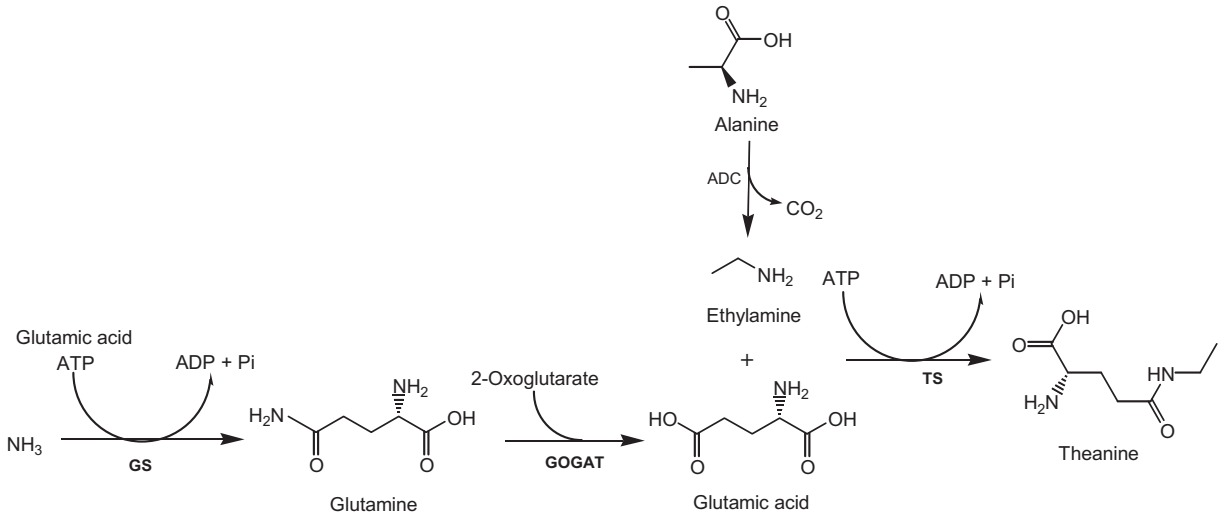

Fig. 5. Possible pathways for theanine biosynthesis in tea seedlings.

content, which reflects the mode of adjustment to salinity stress. And an earlier report showed that the levels of free amino acid increase as a result of salt stress in *B. parviflora* [15]. Our results also suggested that the free amino acids accumulation during NaCl treatment may relate to osmotic adjustment, protection of cellular macromolecules, or the storage form of nitrogen, maintaining cellular pH and detoxification of the cells in the viewpoint of physiology of tea plants.

Furthermore, theanine, the most abundant amino acid in tea seedlings [5], occupies more than 50% in the total free amino acids in this result. It was found in the same variation trend with the total free amino acid contents no matter in tea roots or shoots (Fig. 3). The changes of theanine concentration during salt stress was gradually increased up to the 8 DAT and then declined at 22 DAT in roots, while enhanced by degrees in shoots, which were also illustrated in HPLC-chromatograms for theanine identification (Fig. 4). Due to the previous report by Sasaoka [16], the theanine biosynthetic enzyme required potassium ion to maintain its activity. And high-affinity potassium (K^+) uptake was activated by micromolar sodium (Na^+) concentrations. It is reported that HKT1, high-affinity potassium (K^+) uptake transporter, plays a possible role for Na^+ inhibition of K^+ accumulation in physiological Na^+ toxicity in wheat [17]. The concentration of theanine increased in this study may be due to the intensified protein hydrolysis and also the increased theanine synthetase activity.

Glutamic acid is a direct substrate for theanine biosynthesis, and the changes of its concentration during salt stress were also investigated (Fig. 3). Glutamic acid content was higher in shoots than in roots, and gradually increased by the salt treatment in roots, while it slightly decreased at 1 DAT and then greatly increased (8 DAT) and declined afterward (22 DAT) in shoots. As shown in Fig. 5, alanine can be converted to ethylamine by alanine decarboxylase [18,19], and used for theanine synthesis in tea seedlings [6]. We also surveyed the concentration of alanine under salt treatment on the theanine metabolism. The alanine level increased by nearly 3-fold at 1 DAT, in comparison with no salt treatment as a control, and then enhanced gently in tea roots; but the alanine level gradually increased to 8 DAT then advanced more than 10 times at 22 DAT in shoots. The theanine content increased in this work may also relate to the increased concentrations of these substrates, which are involved in theanine biosynthetic pathway.

4. Conclusion

Theanine biosynthesis derived from nitrogen metabolism in tea plants can be influenced by salt treatment. In this study, we investigated the effect of salt treatment on theanine biosynthesis. Polyclonal antibodies with high sensitivity against TS, obtained from rabbits, were utilized for immunoblotting analysis. We concluded that the expression patterns of TS protein in tea seedlings were gradually increased after salt treatment. The levels of theanine and the total free amino acids were also gradually increased during salt treatment. Our results illustrated the increased theanine concentration may be due to the intensified protein hydrolysis and also the increased TS activity. The changes of glutamic acid and alanine, the direct or indirect substrates for theanine biosynthesis, were also determined during salt treatment. Theanine content increased in this work may also relate to the growing substrates, which are involved in the biosynthetic pathway of theanine. The accumulation of free amino acids during NaCl treatment may related to osmotic adjustment, the protection of cellular macromolecules, the storage form of nitrogen, or maintaining cellular pH and detoxification of the cells in the viewpoint of the physiology of tea plants.

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References

[1] M.M. Chaves, J. Flexas, C. Pinheiro, Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell, Ann. Bot. 103 (2009) 551–560.
[2] H. Ashihara, H. Sano, A. Crozier, Caffeine and related purine alkaloids: biosynthesis, catabolism, function and genetic engineering, Phytochemistry 69 (2008) 841–856.
[3] D. Chu, K. Kobayashi, L.R. Juneja, Yamamoto, Theanine – its synthesis, isolation, and physiological activity, in: T. Yamamoto, L.R. Juneja, D. Chu, M. Kim (Eds.), Chemistry and Applications of Green Tea, CRC Press, Boca Raton, 1997, pp. 129–135.
[4] A. Crozier, T. Yokota, I.B. Jaganath, S.C. Marks, M. Saltmarsh, M.N. Clifford, Secondary metabolites in fruits, vegetables, beverages and other plant-based dietary components, in: A. Crozier, M.N. Clifford, H. Ashihara (Eds.), Plant Secondary Metabolites - Occurrence, Structure and Role in the Human Diet, Blackwell, Oxford, 2006, pp. 208–302.

[5] W.W. Deng, S. Ogita, H. Ashihara, Biosynthesis of theanine (γ-ethylamino-L-glutamic acid) in seedlings of *Camellia sinensis*, Phytochem. Lett. 1 (2008) 115–119.
[6] W.W. Deng, S. Ogita, H. Ashihara, Ethylamine content and theanine biosynthesis in different organs of *Camellia sinensis* seedlings, Z. Naturforsch. 64C (2009) 387–390.
[7] W. Zhou, Q.J. Sun, C.F. Zhang, Y.Z. Yuan, J. Zhang, B.B. Lu, Effect of salt stress on ammonium assimilation enzymes of the roots of rice (*Oryza sativa*) Cultivars differing in salinity resistance, Acta Bot. Sin. 46 (2004) 921–927.
[8] W.W. Deng, H. Ashihara, Profiles of purine metabolism in leaves and roots of *Camellia sinensis* seedlings, Plant Cell Physiol. 51 (2010) 2105–2118.
[9] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant 15 (1962) 473–497.
[10] L. Tian, W.F. Kong, Q.H. Pan, J.C. Zhan, P.F. Wen, J.Y. Chen, S.B. Wan, W.D. Huang, Expression of the chalcone synthase gene from grape and preparation of an anti-CHS antibody, Protein Expr. Purif. 50 (2006) 223–228.
[11] D. Jani, L.S. Meena, Q.M. Rizwan-ul-Haq, Y. Singh, A.K. Sharma, A.K. Tyagi, Expression of cholera toxin B subunit in transgenic tomato plants, Transgenic Res. 11 (2002) 447–454.
[12] T. Tsushida, T. Takeo, Ethylamine content of fresh tea shoots and made tea determined by high performance liquid chromatography, J. Sci. Food Agric. 35 (1984) 77–83.
[13] H. Kotaniguchi, M. Kawakatsu, T. Toyo'oka, K. Imai, Automatic amino acid analysis utilizing 4-fluoro-7-nitrobenzo-2-oxa-1, 3-diazole, J. Chromatogr. 420 (1987) 141–145.
[14] A.K. Parida, A.B. Das, B. Mittra, P. Mohanty, Salt-stress induced alterations in protein profile and protease activity in the mangrove *Bruguiera parviflora*, Z. Naturforsch. 59c (2004) 408–414.
[15] A. Parida, A.B. Das, P. Das, NaCl stress causes changes in photosynthetic pigments, proteins and other metabolic components in the leaves of a true mangrove, *Bruguiera parviflora*, in hydroponic cultures, J. Plant Biol. 45 (2002) 28–36.
[16] K. Sasaoka, M. Kito, Y. Onishi, Some properties of the theanine synthesizing enzyme in tea seedlings, Agric. Biol. Chem. 29 (1965) 984–988.
[17] F. Rubio, W. Gassmann, J.I. Schroeder, Sodium-drive potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance, Science 270 (1995) 1660–1663.
[18] T. Takeo, L-Alanine as a precursor of ethylamine in *Camellia sinensis*, Phytochemistry 13 (1974) 1401–1406.
[19] T. Takeo, L-Alanine decarboxylase in *Camellia sinensis*, Phytochemistry 17 (1978) 313–314.