Plant Growth Regul (2013) 71:295–299
DOI 10.1007/s10725-013-9828-1

BRIEF COMMUNICATION

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

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Received: 14 October 2012 / Accepted: 24 May 2013 / Published online: 5 June 2013

Abstract Theanine synthetase (TS) is a key enzyme involved in theanine biosynthesis. In our recent study, it has been revealed that theanine biosynthesis derived from nitrogen metabolism in tea (*Camellia sinensis*) plants can be influenced by shading treatment. The expression patterns of CsTS protein in the roots and shoots of tea seedlings were examined by western blot using a self-prepared polyclonal antibody with high specificity and sensitivity. The effect of long-term shade treatment on the levels of theanine synthesis was also investigated using roots and shoots of tea seedlings. Levels of theanine and total free amino acids gradually increased in shoots, reaching their maximum after 22 days of treatment (DOT). The immunoblotting analysis suggested that CsTS protein levels increased gradually up to 22 DOT and expression remained at a high level, except after 1 DOT where levels were low in both roots and shoots. The increased theanine concentration we observed in the shading treatment may be due to increased nitrogen assimilation and reduced theanine catabolism under shade conditions.

Keywords *Camellia sinensis* · Theanine synthetase · Shade · Theanine · Amino acid

Introduction

Amino acids in plants are synthesized from primary metabolites. Using carbon and oxygen from the air and hydrogen from water, carbohydrates are synthesized by photosynthesis. Combined with nitrogen obtained from the soil, amino acids can be created by some collateral metabolic pathways. Therefore, photosynthesis plays a vital role in the synthesis of amino acids in plants. Tea (*Camellia sinensis*) is somewhat unique among crop plants as an evergreen shrub whose photosynthesizing leaves are continually harvested. Tea plants are often cultured under shade to improve the quality of plucked leaves (Sakai 1975). Containing active ingredients such as caffeine, catechins and theanine, tea is one of the most important economic plants used in beverages (Crozier et al. 2006). Among these chemicals, theanine is a non-protein amino acid that was first discovered in tea leaves by Sakato (1949). Theanine has been extensively studied in relation to food science and human nutrition because of its unique taste characteristic known as "umami" (Yamaguchi and Ninomiya 2000) and greatly influences tea quality.

Previously, reports have focused on the effect of shading on tea plant growth, photosynthesis, mineral nutrient status and free amino acid accumulation (Sakai 1975; Takeo 1981; Okano et al. 1997). Zhang et al. (2004) reported that the content of amino acids in fresh leaves is increased after shading, from which was concluded that moderate shading could improve the quality and yield of Oolong tea. It was also shown that the tea leaves which were grown in the

Electronic supplementary material The online version of this article (doi:10.1007/s10725-013-9828-1) contains supplementary material, which is available to authorized users.

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shade contain high amino acid but low catechin contents (Ku et al. 2010). In tea cultivation, shadings methods are commonly used to increase the content of L-theanine and enhance the heavy and mellow taste. However, the effect of shade treatment on theanine biosynthesis in tea plants remains unknown.

In this study, changes in the main free amino acids, especially theanine, and the expression of theanine synthetase at the protein was investigated in tea seedlings under shade treatment for 22 days. We demonstrated that the total concentration of major amino acids gradually increased in both roots and shoots, but at a low level after 1 DOT (day of treatment) in roots. Theanine content followed the same trend as total free amino acids, reaching its maximum after 22 DOT. Glutamic acid, a direct precursor for theanine biosynthesis, showed the same trend as theanine. Furthermore, theanine synthetase in the roots and shoots of shade treated plants was investigated at the protein level by generating a specific antibody. Consequently, theanine biosynthesis influenced by shading treatment was discussed in this study.

Materials and methods

Plant materials and shade treatment

Tea (*C. sinensis* cv. Longjing 43) seeds were collected from the tea plantation at the Anhui Agricultural University, Hefei city, Anhui Province, China. To promote germination, the seeds were sterilized with sodium hypochlorite and the seed coats were removed. Seeds were pre-germinated on filter paper, and cultured for 6 months as previously described (Deng and Ashihara 2010). For preparation of experimental materials, no additional nutrients were supplied to the seedlings, which were covered by black shading nets (20 ± 5 % light transmitted) every day. Seedlings of shoots and roots were collected after 1, 8 and 22 DOT and washed with distilled water. Samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

Determination of endogenous amino acid content

Amino acids were extracted and analyzed according to Tsushida and Takeo (1984) with a slight modification as follows. Roots and shoots were immediately placed 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 made up to 100 mL with distilled water. Samples were adjusted to pH 8.0 with 50 mM borate buffer for amino acid analysis. Amino acids, including theanine, were separated and analyzed using a HPLC system with a fluorescent detector adopted for free amino acid analysis (Kotaniguchi et al. 1987). Amino acid standards were obtained from Sigma (St. Louis, MO, USA).

Construction of the pEasy-TS fusion protein expression vector

The coding region of the theanine synthetase (TS) gene (GenBank Accession No. DD410895) was amplified by RT-PCR from total RNA of *C. sinensis* young leaves using gene-specific primers; 5′-GCCAGATCTATGTCTCTTCT TTCCG-3′ (forward, *BglII* site underlined) and 5′-TTGTC GACTTACGGTTTCCAGAGG-3′ (reverse, *SalI* site underlined). The PCR reaction was performed in a 30 μL volume containing 1.6 μg cDNA from tea leaves as template using the LA Taq polymerase system (Takara, Otsu, Japan) for 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 of 10 min at 72 °C. Amplified PCR products were digested with the respective restriction enzymes and cloned in frame with the 6-His tag of the 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 Rosetta Gami (DE3) cells and the recombinant CsTS protein was expressed according to the pEasy-E1 vector manufacturer's instructions (TransGen, Beijing, China).

Expression and purification of the recombinant protein, antibody production and purification

All these methods were performed as described by Tian et al. (2006) and Deng et al. (2012).

SDS–polyacrylamide gel electrophoresis and western analysis

Western blot was performed as described by Tian et al. (2006). The method for extraction of total soluble protein was similar to a previous report (Jani et al. 2002), but modified as follows. Samples (ca. 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 homogenizing, 20 μL phenylmethanesulfonyl fluoride (PMSF) and 40 μL nonylphenoxypoly (ethyleneoxy) ethanol (NP-40) were added. The supernatant was collected and precipitated with 3–5 volumes of 10 % (w/v) trichloroacetic acid in cold (−20 °C) acetone for 2–4 h. After centrifuging, the precipitate was washed with 0.07 % DTT (w/v) in cold (−20 °C) 80 %

acetone. Proteins were dried under vacuum, stored at −20 °C or resuspended 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 centrifuging, the supernatant was collected and stored at −80 °C until use. The amount of total protein was measured with a Bradford protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as a standard. Proteins (10 μg) were separated by SDS-PAGE (10 % resolving gel, 4 % stacking gel) and transferred to polyvinylidene fluoride membranes using a semidry electroblotter (Bio-Rad, California, USA). After blocking for 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 rabbit-anti-TS antibody for 1 h. Following three washes in TBS buffer, membranes were incubated with anti-rabbit (IgG) (Sigma, St. Louis, MO, USA) as secondary antibody (1:5,000 diluted), and complexes were visualized using a detection kit (Thermo, Rockford, USA.). The maximum sensitivity substrate could be determined clearly with 1–100 fg protein. The relative changes in TS protein level were quantified using the Bio-Rad Quantity One software. Experiments were performed in duplicate and repeated at least three times.

Results

The specificity of the antibody preparation was also tested by western blot using the purified TS. Similar result was shown in our previous paper (Deng et al. 2012). The protein level of TS showed similar patterns in both tea roots and shoots during shading treatment. TS protein accumulation was low after 1 DOT and gradually increased (Fig. 1).

In total we determined the content of 17 free amino acids in the roots and shoots of both control and shade treated (1, 8 and 22 days) tea seedlings. The average content of total free amino acids after 0, 1, 8 and 22 DOT was, respectively, 44.60, 41.97, 58.53, 67.22 mg g^{-1} dry weight in roots and 10.77, 18.94, 23.02, 42.68 mg g^{-1} dry weight in shoots (Fig. 2), thus showing total free amino acid content was significantly increased by shading treatment. The maximum content was found after 22 DOT in both roots and shoots. Compared with shoots, the concentration of total free amino acids was much higher in roots, but the degree of increase was much lower. The concentration of total free amino acids at 22 d is about fourfold higher than that of 0 d in shoot, however, the content of the total free amino acids in roots at 22 d has not been doubled.

Discussion

In *Pinus ponderosa* seedlings, an increase in total amino acid concentration was induced by drought and shading (Vance and Zaerr 1990), with shading having the greater effect. This report also revealed that arginine and glutamine were increased the most in the shaded seedlings. The large increase in arginine in severely shaded seedlings suggests that sequestering and storage of ammonia are important when stress reduces carbon fixation.

Our results also showed free amino acid accumulation during the shading treatment. Theanine, the most abundant amino acid in tea seedlings (Deng et al. 2008), occupied a large percentage of the total free amino acids and followed the same trend as the total free amino acids in both roots and shoots (Fig. 2). Okano et al. (1997) states that theanine is first synthesized in roots and then transported via the xylem to the shoot, where it is relatively stable. Theanine concentration during shading treatment gradually increased, and reached a maximum after 8 DOT in roots and 22 DOT in shoots specially, as illustrated in HPLC-chromatograms for theanine identification (Supplementary Data). As shown in the theanine biosynthetic pathway (Fig. 3) glutamic acid is a direct substrate for theanine biosynthesis, thus the changes in its concentration during shading treatment were also examined. Figure 2 shows that glutamic acid content increased in both roots and shoots of shaded tea seedlings.

The *N*-ethyl carbon of theanine is significantly incorporated into the phloroglucinol nucleus of catechin in tea shoots (Kito et al. 1968). This incorporation is controlled by light, which may be one reason for theanine content increasing during shading treatment. On the other side, some evidence has shown that the process of nitrogen assimilation into amino acids is subject to light and metabolic control at the molecular level (Lam et al. 1996). Nitrogen metabolism should be prompted under shade conditions in plants (Sakai 1975; Takeo 1981; Wojciechowska and Siwek 2006). Our Western blot analysis of TS expression could support this hypothesis as the TS protein expression level (Lam et al. 1996) gradually increased under the shading conditions, even though there was a slight decline at day 1 in both tea roots and shoots that did not correspond to the changes in theanine content. Otherwise, the accumulation of TS protein almost completely coincided with the changes in theanine concentration during shading treatment. Therefore, the increased concentration of theanine under shading treatment in this research may be due to increased nitrogen assimilation and reduced theanine catabolism under shade conditions.

In this paper, theanine biosynthesis derived from nitrogen metabolism in tea plants can be influenced by shade treatment. Polyclonal antibodies with high sensitivity

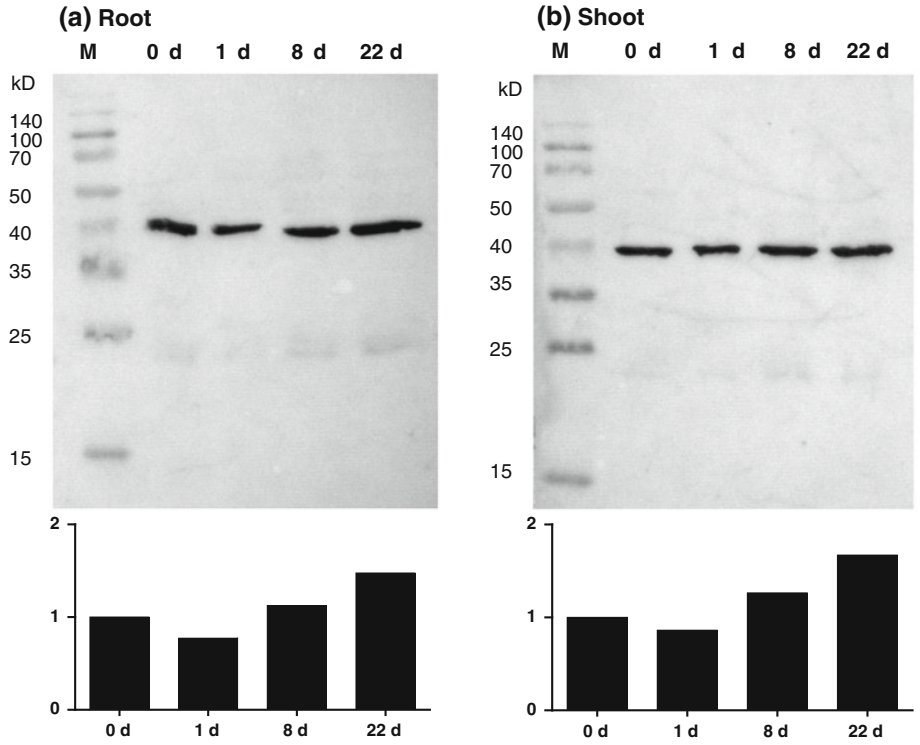

Fig. 1 Western blot analysis of TS expression in roots (**a**) and shoots (**b**) of tea seedlings during shading treatment after 0, 1, 8 and 22 DOT. 10 μg proteins per lane were loaded onto the SDS-PAGE. The *histograms* expressed the relative changes in TS protein levels which quantified by "Bio-Rad Quantity One" software. The Data shown are from one representative experiment of three replicates

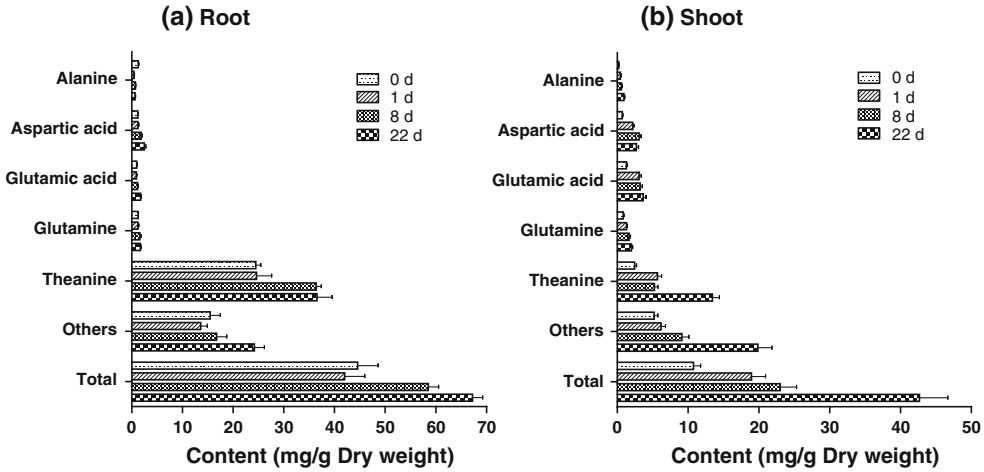

Fig. 2 Concentrations of theanine and other main amino acids in roots (**a**) and shoots (**b**) of tea seedlings during shading treatment after 0, 1, 8 and 22 DOT

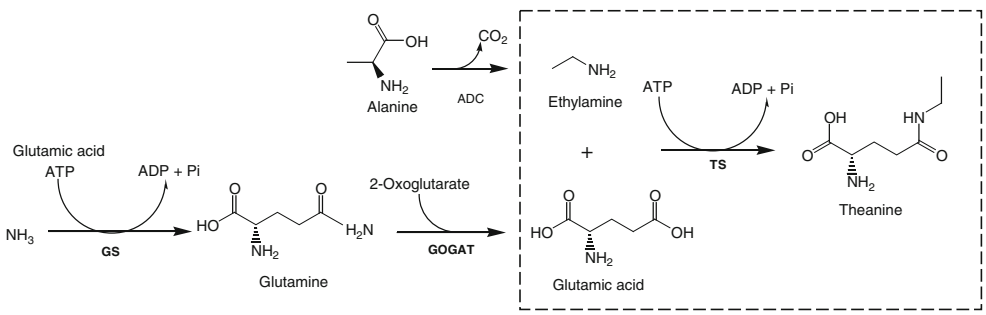

Fig. 3 Possible pathways for theanine biosynthesis in tea seedlings

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 shade treatment. Levels of theanine and total free amino acids gradually increased in both roots and shoots. The increased theanine concentration we observed in the shading treatment may be due to increased nitrogen assimilation and reduced theanine catabolism under shade conditions. In this experiment, 6-month-old tea seedlings were used to determine theanine biosynthesis under shading treatment. And a study of theanine biosynthesis of adult tea trees for production is undergoing, which can be adopted to explain the shading methods for theanine increasing and quality improvement in tea cultivation.

Acknowledgments This research was supported by the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT1101) and Natural Science Foundation of China (No. 31170283).

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