Expression of Caffeine Biosynthesis Genes in Tea (*Camellia sinensis*)

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Using semi-quantitative reverse transcription-PCR, we studied the expression of genes encoding caffeine synthase (*TCS1*), inosine-5′-monophosphate dehydrogenase (*TIDH*), *S*-adenosyl-L-methionine synthase (*sAMS*), phenylalanine ammonia-lyase (*PAL*) and α-tubulin (*Tua1*) in young and mature leaves, stems and roots of 4-month-old tea seedlings and young and old tea tissue cultures. The amounts of transcripts of *TCS1* were much higher in young leaves than in other parts of the plant. Expression of *TIDH* was greater in leaves than in other parts. Little difference in the amounts of transcripts of *PAL*, *sAMS* and *Tua1* was found between various organs of tea seedlings. Larger amounts of transcripts of *TCS1* and *PAL* were found in young callus tissues than in old tissues. These results support our conclusion deriving from previous enzymatic and metabolic studies that caffeine is synthesized mainly in young leaf tissues. We propose that marked caffeine biosynthesis in young leaves is dependent on a greater expression of the *TCS1* gene in the organ.

Key words: Caffeine Biosynthesis, Gene Expression, *Camellia sinensis*

Introduction

In our previous paper, we reported the contents of purine alkaloids in different parts of seedlings and tissue cultures of *Camellia sinensis* (Ashihara and Kubota, 1986). More than 99% of the caffeine detected was in the leaves of 4-month-old seedlings. The amount of caffeine expressed per g fresh weight was greater in older leaves, but theobromine, a precursor of caffeine biosynthesis, was found only in young leaves. Biosynthetic activity, estimated by incorporation of radioactivity from [^{14}C]adenine into purine alkaloids, suggested that caffeine biosynthesis occurred only in young leaves. Subsequently a key enzyme of caffeine biosynthesis, the dual functional *S*-adenosyl-L-methionine (SAM)-dependent *N*-methyltransferase, which catalyzes the final steps of caffeine biosynthesis, was purified from tea leaves and was named caffeine synthase (Kato *et al.*, 1999). After that, a gene encoding this enzyme was cloned and sequenced (Kato *et al.*, 2000). Keya *et al.* (2003) reported that inosine-5′-monophosphate (IMP) dehydrogenase is important in caffeine biosynthesis. The full length cDNA encoding IMP dehydrogenase was also cloned from tea leaves; it was sequenced and named *TIDH* (Keya, 2004).

In the present study, we compared the expression of genes encoding caffeine synthase (*TCS1*), IMP dehydrogenase (*TIDH*), *S*-adenosyl-L-methionine (SAM) synthetase (*sAMS*), phenylalanine ammonia-lyase (*PAL*) and α-tubulin (*Tua1*) in various organs of 4-month-old tea seedlings and in young and old tea tissue cultures.

Materials and Methods

Mature tea seeds (*Camellia sinensis*, cv. Longjing 43) were collected from the tea plantation at Anhui Agricultural University, Hefei, China, in October 2006. The tea seedlings were grown in vermiculite without any additional nutrients under natural light. Young leaves, including apex, mature leaves, stems and roots, were used as experimental materials.

Tissue cultures of tea (*Camellia sinensis*, cv. Yabukita) established initially from young leaves, obtained from the Tokyo Metropolitan Agriculture Experimental Station, Tachikawa, Tokyo, Japan, in May 2005, were grown in modified MS medium (Murashige and Skoog, 1962); the concentration of inorganic elements was reduced to half of the

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original, and 10 μM thidiazuron and 3% sucrose were added. The pH value of the medium was adjusted to 5.7, and 0.3% gellan gum was added as a solidified agent. These tissue cultures were transferred to new media every 3 months. Young (1-month-old) and old (3-month-old) cultures were used in the present study. Total RNA was extracted from various parts of tea seedlings and from callus tissues using a modification of the method of Mita *et al.* (2001). The samples were ground to a powder in liquid nitrogen, and transferred to a tube containing one volume of extraction buffer [0.1 M tris(hydroxymethyl)aminomethane-HCl, pH 8; 10 mM EDTA; 0.1 M LiCl; 1% SDS] preheated at 80 °C. Following the addition of one volume of citrate buffer (0.1 M, pH 4.3)-saturated phenol (preheated at 80 °C) and one volume of chloroform/isoamyl alcohol (24:1), the samples were mixed and centrifuged. The aqueous phase was precipitated with one volume of 4 M LiCl overnight at 4 °C. After centrifuging, the pellet was re-suspended in diethylpyrocarbonate (DEPC)-treated water and precipitated with 0.3 M sodium acetate, pH 5.5, and two volumes of absolute ethanol. RNA was re-suspended and precipitated twice with 70% ethanol and a suspension in water was produced of it. The total RNAs obtained were further treated with DNase I (Promega, Madison, WI, USA). DNA-free total RNA was used for first strand cDNA synthesis, in a reaction mixture (20 μl) containing 50 units of MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and oligo-d(T)16. The PCR reaction mixture (25 μl) contained 60 ng cDNA and 12.5 μl GoTaq Green mastermix (Invitrogen, Carlsbad, CA, USA); 25–35 cycles were carried out with the following program: 95 °C for 30 s, 53 °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 amount of the products was determined densitometrically after gel electrophoresis, to indicate the relative level of transcripts of each gene.

The gene specific primers were prepared from the tea database. The sequences used in this work were as follows: *TCS1* (AB031280), 5′-AGCAAAGCTACCGAAGACCA-3′ and 5′-TCCACACAAGAGCAAAATGC-3′; *sAMS* (AJ277206), 5′-CTTACGCCATTGGTGTTCCT-3′ and 5′-GGCAGCAGTCTTCAAGAACC-3′; *TIDH* (EU106658), 5′-TGCTGAAAGAAGTGGTGTGC-3′ and 5′-TCCCTTTTGTCATTGCTTCC-3′); *PAL* (D26596), 5′-TCCGATCATCGACAAAATCA-3′ and 5′-AGCTCAGAGAATTGGGCAAA-3′; *Tua1* (DQ340766), 5′-TGGGTTCAAGTGTGGAATCA-3′ and 5′-TCCATACCCTCCCCAACATA-3′.

Results and Discussion

Caffeine biosynthesis starts with the methylation of xanthosine by SAM (Ashihara and Crozier, 1999, 2001). The major route of caffeine biosynthesis is the pathway: xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine. The last two steps are catalyzed by caffeine synthase (Kato *et al.*, 1999). Xanthosine, the starting material for the caffeine biosynthetic pathway, is derived from various substrates, but the most plausible intermediate is IMP, even if it is produced in *de novo* purine biosynthesis or by salvage pathways via adenine nucleotides derived from ATP or SAM (Fig. 1). IMP is converted to XMP by IMP dehydrogenase (IMPDH), and xanthosine is then formed by the reaction catalyzed by 5′-nucleotidase. A specific inhibitor of IMPDH markedly reduces caffeine production in tea and coffee leaves (Keya *et al.*, 2003). We investigated the expression of genes encoding caffeine synthase (*TCS1*), IMPDH (*TIDH*) and SAM synthetase (*sAMS*); Fig. 2A shows the results. For comparison, we also examined expression of the genes (*Tua1*) encoding α-tubulin (often used as a reference gene) and phenylalanine ammonia-lyase (*PAL*), a key enzyme of the general biosynthetic pathway of phenylpropanoids which produce various plant secondary metabolites. The transcripts of these five genes were detected in all organs used in this experiment: young leaves, stems and roots. Although *sAMS*, *PAL* and *Tua1* were expressed almost equally, gene expression of *TCS1* and *TIDH* was greatest in the young leaves, including the apex. Little transcript of *TCS1* was found in mature leaves. Previously, we have found that caffeine is synthesized in young leaves of tea seedlings (Ashihara and Kubota, 1986) and in young leaves of flash shoots in tea trees (Fujimori *et al.*, 1991). Consequently, the activity of caffeine biosynthesis in young leaves is primarily controlled by the enzyme expression of *TCS1*. The high expression of *TCS1* coincides with the activities of *N*-methyltransferases of caffeine biosynthesis in leaves of tea trees. These results are consistent

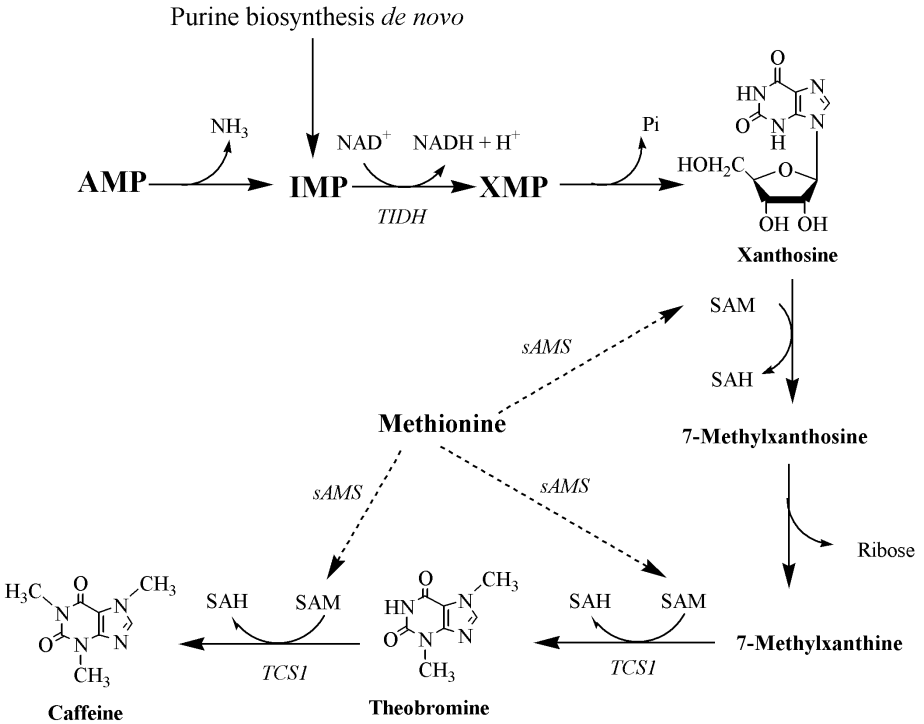

Fig. 1. Caffeine biosynthetic pathway in tea plants, showing the genes studied in this work. IMP, inosine-5′-monophosphate; XMP, xanthosine-5′-monophosphate; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocystein.

with the view that biosynthesis of caffeine in young tea leaves is dependent on the gene expression of *TCS1* and synthesis of caffeine synthase.

Expression of *TIDH* was stronger in young and mature tea leaves than in other organs. IMPDH is involved in the caffeine biosynthesis from adenine nucleotides (Keya *et al.*, 2003; Keya, 2004) as well as the branch of the *de novo* purine biosynthetic pathway leading to GMP (Ashihara and Crozier, 1999; Stasolla *et al.*, 2003; Zrenner *et al.*, 2006). Most genes of purine biosynthesis *de novo* are expressed at lower levels in a constitutive manner in tobacco plants. This observation suggests that these genes have housekeeping functions (van der Graaff *et al.*, 2004). However, some genes, including *IMPDH*, are expressed specifically in sink leaves and floral organs (van der Graaff *et al.*, 2004). The differing expression pattern of *IMPDH* in different organs in tobacco and tea plants suggests that the *IMPDH* gene is involved not only in GMP synthesis but has multiple other functions, such as caffeine biosynthesis in tea.

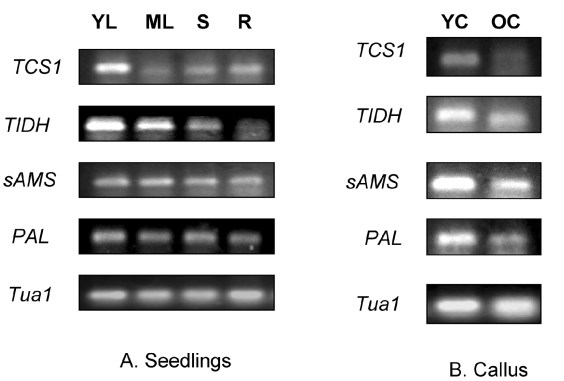

Fig. 2. Expression of *TCS1*, *TIDH*, *sAMS*, *PAL* and *Tua1* in 4-month-old seedlings (A) and tissue cultures derived from leaves (B) of *Camellia sinensis*. (A) YL, young leaves including apex; ML, mature leaves; S, stems; R, roots. The results shown were obtained with 25 (*TCS1* and *Tua1*), 28 (*TIDH* and *sAMS*) and 30 cycles (*PAL*) of PCR amplification. (B) YC, young callus; OC, old callus. The results shown were obtained with 28 (*TCS1*, *TIDH*, *sAMS* and *Tua1*) and 30 cycles (*PAL*) of PCR amplification.

The transcripts of *sAMS* and *PAL* were similarly expressed in all tea organs. SAM is utilized for reactions catalyzed by various *C*-, *N*-, and *O*-methyltransferases in tea plants, although this is an essential co-substrate for caffeine biosynthesis. The pattern of gene expression of *sAMS* may therefore not be synchronized with that of *TCS1*. Expression of *PAL*, which is a gene for a key enzyme of another secondary metabolism pathway, also has a pattern different from the expression of *TCS1*.

We also examined the expression of these five genes in caffeine-producing callus tissues (Fig. 2B). Larger amounts of the transcripts of *TCS1* and *PAL* were found in young callus tissues than in old tissues. Similar trends were also found for *TIDH* and *sAMS*, although the differences were small. These findings also suggest that caffeine biosynthesis is active in young tissues even in tissue cultures. Our earlier conclusion, prior to the discovery of the *TCS1* gene, namely that caffeine biosynthesis occurs in young tissues and mainly in leaves (Ashihara and Kubota, 1986; Fujimori *et al.*, 1991), has been confirmed by the gene expression level of the present study.

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