The first committed step reaction of caffeine biosynthesis: 7-methylxanthosine synthase is closely homologous to caffeine synthases in coffee (*Coffea arabica* L.)[1]

Kouichi Mizuno[a,*], Misako Kato[b], Fumi Irino[b], Naho Yoneyama[b], Tatsuhito Fujimura[c], Hiroshi Ashihara[d]

[a]*Faculty of Bioresource Sciences, Akita Prefectural University, Akita City, Akita 010-0195, Japan*
[b]*Graduate School of Humanities and Sciences, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[c]*Institute of Agricultural and Forest Engineering, University of Tsukuba, Tsukuba Science City, Ibaraki 305-8572, Japan*
[d]*Department of Biology, Faculty of Science, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan*

Abstract In coffee and tea plants, caffeine is synthesized from xanthosine via a pathway that has three methylation steps. We identified and characterized the gene encoding the enzyme for the first methylation step of caffeine biosynthesis. The full-length cDNA of coffee tentative caffeine synthase 1, *CtCS1*, previously isolated by the rapid amplification of cDNA ends was translated with an *Escherichia coli* expression system and the resultant recombinant protein was purified using Ni-NTA column. The protein renamed CmXRS1 has 7-methylxanthine synthase (xanthosine:*S*-adenosyl-L-methionine methyltransferase) activity. CmXRS1 was specific for xanthosine and xanthosine 5′-monophosphate (XMP) could not be used as a substrate. The K_m value for xanthosine was 73.7 µM. *CmXRS1* is homologous to coffee genes encoding enzymes for the second and third methylation steps of caffeine biosynthesis.
© 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Coffee; Caffeine; *N*-methyltransferase; 7-Methylxanthosine; *S*-adenosyl-L-methionine; Xanthosine

1. Introduction

We recently proposed the major biosynthetic pathways of the purine alkaloids in leaves of coffee (*Coffea arabica*) and tea (*Camellia sinensis*) [1–4], but the metabolic pathway of purines, including caffeine, has not been elucidated in detail. The available data indicate that a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway is the major route to caffeine [3,4]. However, xanthosine 5′-monophosphate (XMP), rather than xanthosine, has also

been proposed as the methyl acceptor in coffee leaves [5,6] (Fig. 1).

Since the caffeine biosynthetic pathway contains three *S*-adenosylmethionine (SAM)-dependent methylation steps and a nucleosidase step, *N*-methyltransferases are important for caffeine synthesis (Fig. 1). Previously, we purified caffeine synthases (CS) catalyzing the second and third methylation steps [7], and cloned a gene encoding CS from young tea leaves [8]. Several coffee tentative caffeine synthases (*CtCS*) such as coffee theobromine synthase 1 (*CTS1*), *CTS2* [9] and *CCS1* [10] have been cloned. *CTS1* and *CTS2* are homologous genes encoding theobromine synthases from young coffee leaves [9]. Independently Ogawa et al. made a clone using the sequence of our tea caffeine synthase (*TCS*), *CaMXMT* [11]. In contrast to TCS1, which catalyzes two final methylation steps of caffeine biosynthesis, both from 7-methylxanthine to theobromine and from theobromine to caffeine, CTSs only catalyze the second methylation step, that is, the conversion from 7-methylxanthine to theobromine and do not have 1-*N*-methylation activity (Fig. 1). Recently, we isolated a gene encoding 1-*N*-methyltransferase from coffee, named coffee caffeine synthase 1 (*CCS1*), as homologs to *CTS1* and *CTS2*. CCS1, like TCS1, can catalyze the second and the third methylation steps [10].

Caffeine biosynthesis in coffee is presumed to begin with a 7-*N*-methyltransferase converting xanthosine to 7-methylxanthosine or XMP to 7-methyl XMP. However, no gene(s) encoding the enzyme for the first methylation step have been obtained yet. Although Moisyadi et al. [12] reported cloning of a cDNA encoding xanthosine 7-*N*-methyltransferase. The cDNA sequence, which encodes a 371 amino acid protein, has been shown to be similar to that for a lipase and recombinants had no methyltransferase activity (Mizuno, unpublished data). Here we examined whether or not the previously cloned CtCS series contains the first *N*-methyltransferase. CtCS1 in the CtCS series had activity of converting xanthosine to 7-methylxanthosine. This is the first report on the cloning of the first *N*-methyltransferase of caffeine biosynthesis in coffee, the first key enzyme for the biosynthesis of purine alkaloids from primary purine metabolism. Furthermore, production of transgenic coffee plants with suppressed expression of this gene seems to be a more useful method for preparing decaffeinated coffee beans than previously reported genes such as

*Corresponding author. Fax: (81)-18-872 1677.
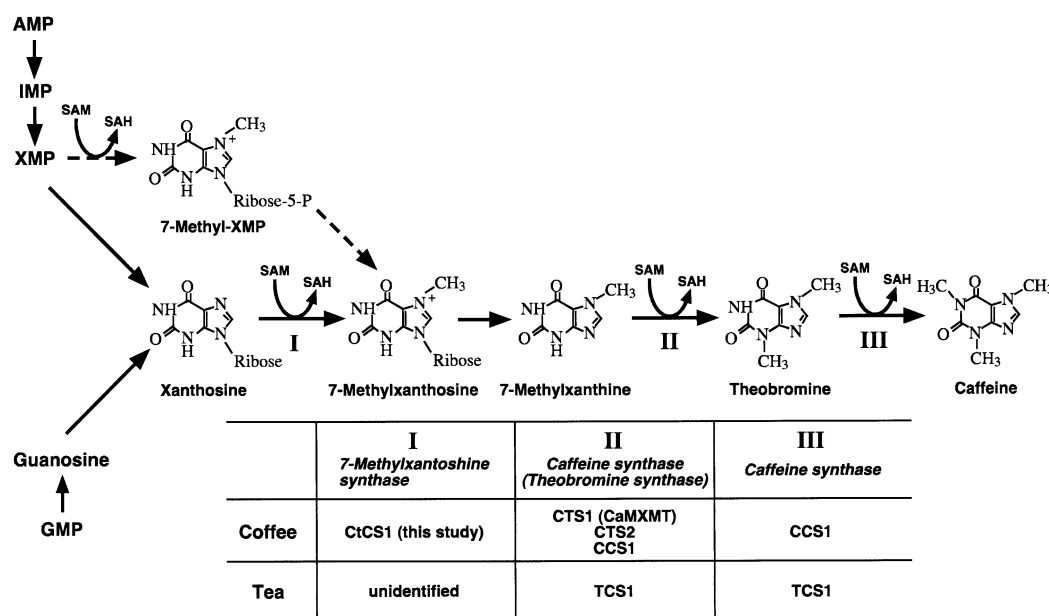*E-mail address:* koumno@akita-pu.ac.jp (K. Mizuno).

Fig. 1. Pathways for the biosynthesis of caffeine. Abbreviations: GMP, guanosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate; IMP, inosine 5′-monophosphate; AMP, adenosine 5′-monophosphate; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine. A XMP→7-methyl XMP→7-methylxanthosine pathway has been proposed in coffee leaves [6,14]. CtCS1 and CTSs have accurate substrate specificity and are produced from xanthosine to 7-methylxantosine (reaction I) and from 7-methylxanthine to theobromine (reaction II), respectively. CCS1 has broad substrate specificity and catalyzes the conversion of 7-methylxanthine to caffeine via theobromine (reactions II and III) [7]. No mXRS gene has been identified from tea.

CCS1. Coffee plants have an active system of degrading xanthosine to CO_2 and ammonia by the entire purine catabolic pathway [3]. Thus xanthosine accumulated in the transgenic plants may be easily removed.

2. Materials and methods

2.1. Materials
Coffee plants (*C. arabica* L.) were grown at 25–30°C in a greenhouse with natural light at the Agricultural and Forest Research Center, University of Tsukuba, Japan. Immature seeds and young leaves were harvested and then stored at −80°C until use. [Methyl-14C]SAM (55 mCi/mmol) was purchased from Amersham Biosciences.

2.2. Cloning of CtCS cDNA
Total cellular RNAs were extracted from coffee tissues in a cetyltrimethylammonium bromide (CTAB) solution according to the method of Chang et al. [13] with exception for using 2-mercaptoethanol at 5%. A cDNA library was constructed from young coffee leaves. cDNA was cloned by screening of the library and by rapid amplification of cDNA ends (RACE) as described previously [9,10].

2.3. Construction of expression plasmids
Plasmids for expressing CtCS1 in *Escherichia coli* were constructed in pET-32a and pET-23d vectors (Novagen) previously [9,10]. Recombinant CtCS1, CtCS3 and CtCS4 proteins from pET32a Trx fusion expression vector were produced in *E. coli* BL21(DE3). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequent Western blot analysis using an anti-CTS2 antibody showed that these recombinant proteins were produced in soluble protein fractions [10]. As the pET-23d vector carries an optional C-terminal His-Tag sequence, the 3′-termination site of *CtCS1* cDNA was replaced by a *Xho*I restriction site using polymerase chain reaction (PCR)-directed mutagenesis and a new expression plasmid for CtCS1 carrying the C-terminal His-Tag (named *CtCS1*:His) was constructed. The primer carrying the *Xho*I restriction site (bold-faced type) was CS-CT1: 5′-**GCTCGAG**CACGTCTGACTTCTCTGG-3′. The primer, named N1, containing initiation condon was also used [10]. The pair of primers was N1/CS-CT1 for CtCS1. The reaction program consisted of 30 cycles at 94°C for 60 s, 55°C for 60 s, and

72°C for 90 s. The PCR product was subcloned into pGEM-T Easy vector (Promega). The subclone of the PCR product for *CmXRS1* was digested with *Nco*I and *Xho*I. The *Nco*I/*Xho*I fragment DNA was introduced into the pET-23d vector at the *Nco*I and *Xho*I sites. The resulting expression plasmids were introduced into the expression host *E. coli* BL21(DE3).

2.4. Production of recombinant enzymes in E. coli and assay for enzyme activity
A single colony of the transformants was cultured at 37°C overnight in 3 ml of Luria broth containing 0.2 mg/ml ampicillin (LA) with constant shaking. A portion (1 ml) of the bacterial culture was added to 50 ml of fresh LA, and incubated at 37°C for 2 h with shaking. Production of the recombinant proteins was induced by adding 150 μl of 0.1 M isopropyl-β-D-thio-galactopyranoside (final concentration is 0.3 mM), and cells were kept at 25°C for 8 h. *E. coli* cells were harvested by centrifugation at $500 \times g$ for 5 min, and then washed with 20 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl (PBS). This cell paste was suspended in a 1 ml of PBS, frozen at −80°C, sonicated to disrupt cells and then centrifuged at $10\,000 \times g$ for 10 min at 4°C. The supernatant was applied to a Ni-NTA column (1 ml) equilibrated previously with PBS containing 10 mM imidazole. After washing with PBS, proteins were eluted by PBS containing 200 mM imidazole. The fractions were used in the enzyme assay for caffeine metabolism. Enzymatic activity of mXRS and CS was determined as described previously [7]. The K_m value was derived from Lineweaver–Burk plots analyzed with a Macintosh G3 computer using 'Enzyme Kinetics' software (Trinity Software, Campton, NH, USA). Thin layer chromatography (TLC) was performed as described [6,14] except that we used *n*-butanol/acetic acid/water (4:1:1, v/v/v) as the solvent.

2.5. Analysis of gene expression by semi-quantitative reverse transcription (RT)-PCR
For semi-quantitative RT-PCR, total RNAs extracted from developing endosperms, flower buds and young leaves of coffee were treated with RNase-free DNase I (TaKaRa). DNA-free total RNA (382 ng) from each tissue was used for first strand cDNA synthesis in 20 μl reaction volume containing 2.5 units of AMV reverse transcriptase XL (TaKaRa) and 1 μM of *oligo-dT 3SAP*. The PCR reaction mixture (20 μl) contained 0.1 μl of RT reaction mixture, 2.5 units of Gene Taq (Nippon Gene), 2.5 mM $MgCl_2$, 2 mM each of deoxyribo-
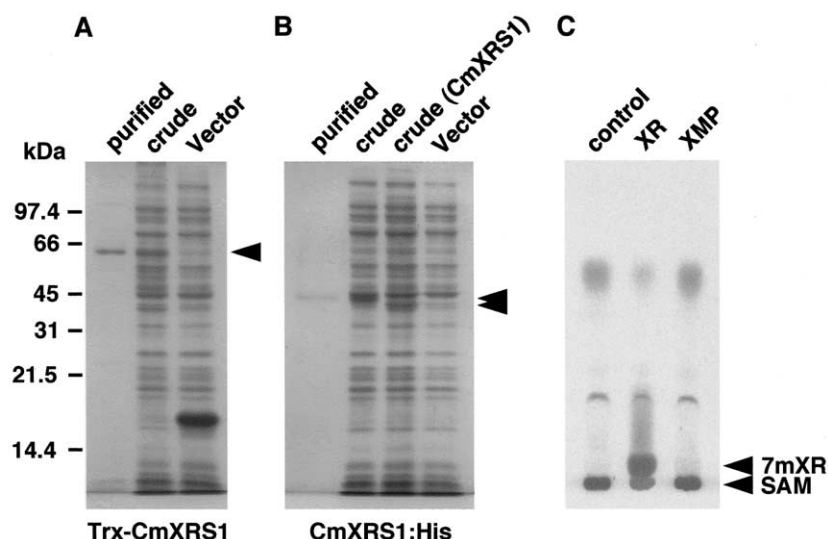
Fig. 2. SDS–PAGE of recombinant CmXRS1 (equal CtCS1) proteins and visualized reaction products derived from recombinant CmXRS1. Soluble protein fractions from *E. coli* cells and its proteins purified by Ni-NTA column were subjected to SDS–PAGE. They were transformed by expression plasmids, derived from pET32a (A: Trx-CmXRS1) or pET23d (B: CmXRS1:His), for CmXRS1. In B, 'crude (CmXRS1)' indicates crude extract from a previous construct (His-Tag less) using pET23d. Gels were stained with Coomassie brilliant blue R-250. C: The autoradiograph of a TLC separation of the reaction products derived from recombinant proteins of purified CmXRS1:His. Products of the reaction mixture were subjected to TLC on a cellulose plate using a solvent system of *n*-butanol/acetic acid/water (4:1:1, v/v/v). Autoradiography was conducted using an Image-Analyzer system (FLA-2000 Fuji-film Co.). XMP and XR indicated substrates. Control, no substrate; XR, xanthosine; 7mXR, 7-methylxanthosine.

nucleotide triphosphate and 0.5 μM of gene-specific primers. The gene-specific primers for *CtCS1* used were 5′-GAATTAGACGCC-CGGAATG-3′ and 5′-CTGCTGAAGGTATATAGAC-3′. A set of specific primers (5′-GCTTTCAACACCTTCTTCAG-3′ and 5′-GCT-GCTCAGGGTGGAAGAG-3′) for the α-tubulin (accession number AF363630) was used for control reaction. For PCR, we used a PTC-200 thermal cycler (MJ Research) programmed at 94°C for 1 min, 57°C for 30 s, and 72°C for 1 min. The amplification was done for 18–30 cycles and reaction tubes were removed every two cycles. The amplicons corresponding to *CtCS1* and α-tubulin were 122 and 133 bp in length, respectively. The amplification showed a linear curve. The reaction product was visualized by ultraviolet (UV) light on 5% polyacrylamide gels stained with ethidium bromide. The intensity of fluorescence was quantitated with a Macintosh computer using 'NIH image' software (http://rsb.info.nih.gov/nih-image/).

2.6. Analytical procedures

Protein concentrations were measured by the method of Bradford [15]. PAGE 0.1% SDS was carried out by the method of Laemmli [16]. Nucleotide sequencing was carried out by the dideoxy chain-termination method of Sanger et al. [17] using a Perkin Elmer genetic analyzer (model 310) and an ABI Prism dye terminator cycle sequencing kit (Perkin Elmer). Nucleotide and protein sequences were analyzed by computer using GENETYX software (Software Development Co., Tokyo).

3. Results and discussion

3.1. Properties of recombinant enzyme

The full-length cDNAs of *CtCS1*, *CtCS3* and *CtCS4* were previously isolated by the RACE method and screening of the cDNA library [9,10]. *CtCS1*, *CtCS3* and *CtCS4* (accession numbers AB034699, AB054842 and AB054843) have 1307, 1373 and 1402 nucleotides, and encode 372, 385 and 385 amino acid residues, respectively. The expression plasmids for the CtCS series cDNA were already constructed in pET-23d and pET-32a (Novagen) vectors [9,10]. The *CtCS1*, *CtCS3* and *CtCS4* cDNAs were translated with an *E. coli* expression system, to elucidate whether the proteins encoded by the cloned

cDNAs are able to catalyze the reaction for the caffeine biosynthetic pathway. Since the activities of the recombinant proteins of CtCS1, CtCS3 and CtCS4 could not be detected in previous experiments, we purified them. Then we re-evaluated the activities with the purified Trx-fused proteins. The crude extracts of these *E. coli* were purified using an Ni-NTA column (Fig. 2A), and the purified proteins were incubated with a variety of xanthine substrates of caffeine in the presence of [methyl-^{14}C]SAM as a methyl donor. CtCS1 could catalyze only the 7-*N*-methylation of the purine ring of xanthosine (Fig. 1). XMP did not show 7-*N*-methylation activity. Although the recombinant CtCS3 and CtCS4 were produced in the soluble extract from *E. coli* and purified, they showed no activity for caffeine intermediates in this assay system. Since CtCS1 catalyzes 7-*N*-methylation of the purine ring to convert xanthosine to 7-methylxanthosine, this clone (*CtCS1*) was renamed coffee 7-methylxanthosine synthase 1 (*CmXRS1*). As described previously, the K_m value of CCS1 for 7-methylxanthine was different from that of the native form (126 μM) or that of the Trx-fused form (75.1 μM) [10]. The difference in the K_m values may be due to the different structure of these recombinant proteins [10]. Therefore, we estimated the enzymatic properties of CmXRS1 using the native form of recombinant protein from the pET23d system.

Table 1
Kinetic parameters of CmXRS1 for xanthine derivatives as substrates[a]

Substrates	K_m (μM)	V_{max} (pkat/mg)	V_{max}/K_m (pkat/mg/μM)
XR	73.7	11.8	0.160
XMP	nd	nd	nd
7mX	nd	nd	nd
Tb	nd	nd	nd

nd, not detected.
[a]Kinetic parameters were estimated by Lineweaver–Burk plot.

However the activity was not detected from the crude extract of the recombinant CmXRS1, there was need to purify the recombinant protein. As the recombinant protein from the previous pET23d expression system for *CmXRS1* could not be purified simply, we reconstructed a new expression plasmid for *CmXRS1* colocated with histidine-tag at the C-terminus (named *CmXRS1*:His). SDS–PAGE showed that the recombinant protein was produced in the soluble protein fraction and was purified by passage through an Ni-NTA column (Fig. 2B).

By separating the methylated products by TLC, we found that CmXRS1 only catalyzes the 7-*N*-methylation activity of xanthosine to 7-methylxanthosine, and does not have 1-*N*- or 3-*N*-methylation activity (Fig. 2C). The kinetic parameters of CmXRS1 are shown in Table 1. The specific activity of recombinant CmXRS1 was 10.6 pkat/mg proteins with xanthosine. The value was much higher than that (1.36 fkat/mg) obtained for NMT activity in the purified enzyme preparation from the liquid endosperm of *C. arabica* seed [18]. The K_m values of caffeine synthetic enzymes for xanthine derivatives as substrates are summarized in Table 2. The K_m value of CmXRS1 for xanthosine was 73.7 μM. Although the K_m value for xanthosine in the mXRS observed in the crude enzyme preparation from tea leaves was 250 μM [20], the value of the native enzyme from coffee plants is unclear. When the K_m values of CTS2 and CCS1 for 7-methylxanthine (171 μM) and theobromine (157 μM), respectively, are considered, the value of CmXRS1 corresponds to the in vivo level. The activity of CmXRS1 examined in 0.1 M sodium phosphate buffer (pH 6.0–7.0) or 0.1 M Tris–HCl buffer (pH 7.0–9.0) at 27°C showed an optimum at pH 7.0, which is similar to that of the corresponding native enzyme from tea leaves [20]. The activities of CTS2 and TCS1 showed an optimum at pH 8.0 and 8.5, respectively (unpublished data).

The present results suggest that at least two different *N*-methyltransferases are utilized for caffeine biosynthesis in coffee plants. CmXRS1 seems to be a key enzyme in the caffeine biosynthetic pathway, because it is located at the branching point for caffeine biosynthesis from the general purine nucleoside and nucleotide metabolism. Two different reactions on the initial methylation step of caffeine biosynthesis have been proposed. Negishi et al. [20,21] found xanthosine 7-*N*-methyltransferase in tea and coffee leaves and proposed a xanthosine pathway of caffeine biosynthesis, which started by the methylation of xanthosine. This pathway has been confirmed by other investigators [3]. By contrast, Baumann and his co-workers proposed a new XMP pathway starting by the methylation of XMP [6]. The present results

Table 2
K_m values of caffeine synthetic enzymes for xanthine derivatives as substrates[a]

Caffeine synthetic enzymes	Substrates (μM)			Reference
	XR	7mX	Tb	
CmXRS1 (recombinant; +His-Tag)	73.7	nd	nd	this study
CTS1 (recombinant; native form)	nd	873	nd	[9,10]
CTS2 (recombinant; native form)	nd	171	nd	[9,10]
CCS1 (recombinant; native form)	nd	126	157	[10]
Young leaves (crude extract)	–	385–390	–	[19]
TCS1 (recombinant; native form)	nd	186	344	[8]

nd, not detected; –, not determined.
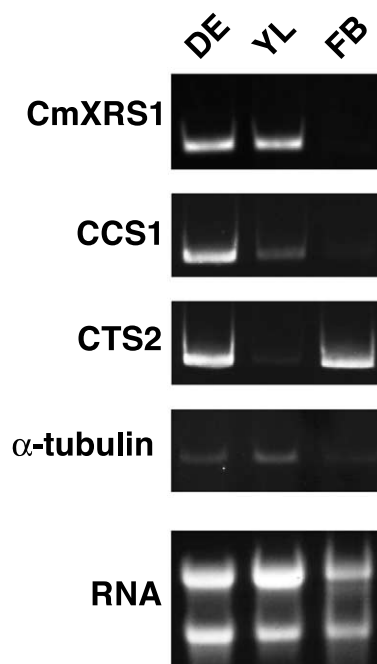[a]K_m values were estimated by Lineweaver–Burk plot.

Fig. 3. Expression of *CmXRS1* in coffee tissues. RT-PCR for *CmXRS1* was performed with 18–30 cycles to determine the linear range of PCR amplifications. Here, the results obtained with 26 cycles for all templates and genes are shown. The quality and concentration of total RNA from each tissue were estimated by ethidium bromide staining. DE, developing endosperms; YL, young leaves; FB, flower buds.

support the presence of the former pathway, although we cannot exclude the possibility that unidentified *CtCSs* are related to the latter pathway, i.e. methylation of XMP.

3.2. Expression of CmXRS1

Expression of *CtCSs* has been demonstrated in young coffee leaves, flower buds and developing fruits by Northern blot analysis of total RNAs from those caffeine-containing tissues [10]. The CtCS cDNAs (*CmXRS1*, *CtCS3*, *CtCS4*, *CtCS7*, *CTS1*, *CTS2* and *CCS1*) cannot be distinguished, because they are over 80% identical to each other at the nucleotide level. As the amount of transcripts from these genes cannot be detected by Northern blot analysis, the expression of *CmXRS1* was evaluated by semi-quantitative RT-PCR (Fig. 3) designed for specific amplification and detection of these transcripts. Although the transcripts of *CmXRS1* were detected in all organs used in this experiment (developing endosperm, young leaf, and flower bud), the gene expression was the strongest in the developing endosperm. We also examined the expression patterns by RT-PCR for the transcripts of *CTS2* and *CCS1* [10]. *CTS2* and *CCS1* were expressed in all organs and the amount of transcripts was the highest in the developing endosperm like that of *CmXRS1*. Although *CTS2* was expressed strongly in flower buds, the expression of *CmXRS1* and *CCS1* was not so strong. Moreover a smaller amount of *CTS2* transcripts was detected in young leaves than those of *CmXRS1* and *CCS1*. Although the expression patterns of *CmXRS1* and *CCS1* were synchronized, that of *CTS2* was not. These findings may indicate that different caffeine synthetic pathways are involved in the differential expression of *CmXRS1* and *CTS2* in the young leaves and endosperms of the coffee plant.
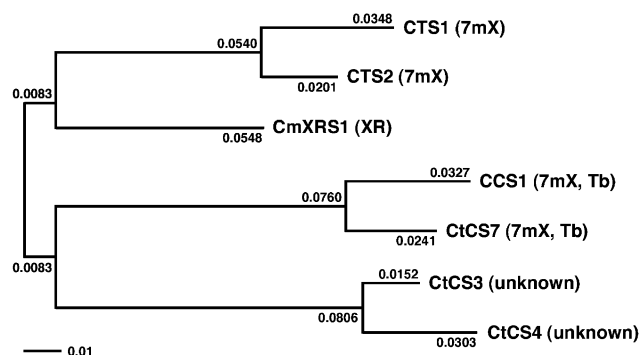
Fig. 4. A phylogenetic tree analysis of coffee CtCS series. The tree for coffee CtCS series was constructed by the neighbor-joining program with GENETYX (Software Development Co., Tokyo, Japan). Substrates of the enzymes are indicated in parentheses. Abbreviations of substrates are as follows: XR, xanthosine; 7mX, 7-methylxanthine; Tb, theobromine. Sources of the sequences are as follows: *CmXRS1*, AB034699; *CCS1*, AB086414; *CtCS7*, AB086415; *CTS1*, AB034700; *CTS2*, AB057412; *CtCS3*, AB054842; *CtCS4*, AB054843.

3.3. Comparison of the sequences of CmXRS1 and other N-methyltransferases

CmXRS1 has only the activity of 7-*N*-methylation of xanthosine. All proteins catalyzing *N*-methylation in the caffeine synthesis pathway from coffee, including the products of *CmXRS1* and other *CtCSs*, are highly homologous (>80%) with each other, but only CmXRS1 has this activity. On the contrary, TCS1 also has this enzymatic activity although TCS1 has only an overall 40% homology to CCS1. These proteins including TCS1 share four highly conserved regions, motif A, motif B′, motif C and YFFF region [10] with slight deviations. These regions may have important roles both in the common *N*-methylating catalytic reaction and in discriminating the positions of *N*-methylation of the purine ring. Motif A is speculated to be a SAM-binding site [22,23] which commonly plays an important role in these proteins. When the amino acid sequence of CmXRS1 was compared with those of the other CtCS series, CmXRS1 lacked the 13-residue sequence at the C-terminal region [10]. The molecular size of the substrate of CmXRS1, xanthosine, in which xanthine is combined with ribose, obviously differs from those of 7-methylxanthine and theobromine which are the substrates of CTS and CCS, respectively. The site for a 13-residue sequence, lacking from CmXRS1, probably locates the substrate-binding region closely, and forms a space for binding the ribose of xanthosine as a substrate. However, since such specificity was not found in TCS1, it is suggested that the caffeine synthetic pathway evolved in parallel in tea and coffee. In order to obtain evidence for the parallel evolution of caffeine synthetic pathways, we are performing experiments to isolate and compare the genes derived from other caffeine-containing plants, i.e. *Theobroma cacao* (cocoa) and *Ilex paraguayensis* (mate). A phylogenetic tree of coffee CtCS series was created, aligning

the enzymes by the substrate specificities (Fig. 4). CtCS3 and CtCS4, the activities of which are unknown, formed a branch separated from the other CtCSs (Fig. 4). CtCS3 and CtCS4 may have caffeine activity and/or its derived metabolic activity previously unknown.

CmXRS1, which is the key enzyme catalyzing the first step in the methylation of caffeine biosynthesis, is considered to be useful for production of naturally caffeine-free coffee. Interestingly, the substrate and product specificities of CtCS series are different, though the genes are highly homologous (>80%) with each other. We will investigate this matter using site-directed mutagenesis and chimeric enzymes.

References

[1] Ashihara, H., Monteiro, A.M., Gillies, F.M. and Crozier, A. (1996) Plant Physiol. 111, 747–753.
[2] Ashihara, H., Gillies, F.M. and Crozier, A. (1997) Plant Cell Physiol. 38, 413–419.
[3] Ashihara, H. and Crozier, A. (1999) Adv. Bot. Res. 30, 117–205.
[4] Ashihara, H. and Crozier, A. (2001) Trends Plant Sci. 6, 407–413.
[5] Nazario, G.M. and Lovatt, C.J. (1993) Plant Physiol. 103, 1203–1210.
[6] Schulthess, B.H., Morath, P. and Baumann, T.W. (1996) Phytochemistry 41, 169–175.
[7] Kato, M., Mizuno, K., Fujimura, T., Iwama, M., Irie, M., Crozier, A. and Ashihara, H. (1999) Plant Physiol. 120, 579–586.
[8] Kato, M., Mizuno, K., Crozier, A., Fujimura, T. and Ashihara, H. (2000) Nature 406, 956–957.
[9] Mizuno, K., Tanaka, H., Kato, M., Ashihara, H. and Fujimura, T. (2001) in: International Scientific Colloquium on Coffee, Vol. 19, pp. 815–818, ASIC, Paris.
[10] Mizuno, K., Okuda, A., Kato, M., Yoneyama, N., Tanaka, H., Ashihara, H. and Fujimura, T. (2003) FEBS Lett. 534, 75–81.
[11] Ogawa, M., Herai, Y., Koizumi, N., Kusano, T. and Sano, H. (2001) J. Biol. Chem. 276, 8213–8218.
[12] Moisyadi, S., Neupane, K.R. and Stiles, J.I. (1998) Acta Hort. 461, 367–376.
[13] Chang, S., Puryear, J. and Cairney, J. (1993) Plant Mol. Biol. Rep. 11, 113–116.
[14] Kato, M., Kanehara, T., Shimizu, H., Suzuki, T., Gillies, F.M., Crozier, A. and Ashihara, H. (1996) Physiol. Plant 98, 629–636.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Laemmli, U.K. (1970) Nature 272, 680–685.
[17] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[18] Mazzafera, P., Wingsle, G., Olsson, O. and Sandberg, G. (1994) Phytochemistry 37, 1577–1584.
[19] Mösli Waldhauser, S.S., Gillies, F.M., Crozier, A. and Baumann, T.W. (1997) Phytochemistry 45, 1407–1414.
[20] Negishi, O., Ozawa, T. and Imagawa, H. (1985) Agric. Biol. Chem. 49, 2221–2222.
[21] Negishi, O., Ozawa, T. and Imagawa, H. (1985) Agric. Biol. Chem. 49, 887–890.
[22] Kagan, R.M. and Clarke, S. (1994) Arch. Biochem. Biophys. 310, 417–427.
[23] Joshi, C.P. and Chiang, V.L. (1998) Plant Mol. Biol. 37, 663–674.